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SOME INVESTIGATIONS ON CHOLINE METABOLISM IN ASPERGILLUS NIGER

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CHAPTER I

LITERATURE SURVEY REGARDING CHOLINE

1. INTRODUCTION

Choline is one of the most important nitrogen compounds of the non-protein fraction of plants and animals. Synthesis and dissimilation of free choline form an integrating part of the metabolism of compounds with labile methylgroups. Acetylcholine and glycerophosphoric acid ester of choline belong to the watersoluble derivatives of choline; lecithin and sphingomyeline are choline containing compounds in fats.

Choline metabolism in plants has been only superficially investigated. With regard to synthesis HOROWITZ' work is of special importance (63, 64). By comparing the growth factors of a number of choline-less mutants of *Neurospora crassa* they were able to prove that the synthesis of choline is the result of the methylation of ethanolamine step by step.

The origin of the methylgroups of choline has been investigated by KIRKWOOD c.s. (99); they isolated choline with C^{14} in the methylgroups after feeding $NaC^{14}OOH$ to sprouting barley.

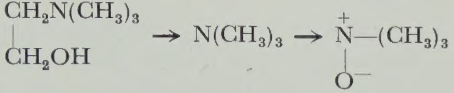
The greatest part of the experiments on the metabolism of choline has been made in animals (DU VIGNEAUD, BORSOOK, DUBNOFF); They proved that in rats methionine can provide a methylgroup for the synthesis of choline, whereas conversely choline can function indirectly as a methyldonor at the synthesis of methionine (140). This combined action of compounds with "biological labile methylgroups" is jointly termed transmethylation reactions.

During the dissimilation choline can be oxidised to betaine under the influence of a choline oxidising enzymsystem; the degradation of choline to trimethylamine has been shown too. The object of the investigation, the results of which have been described here, was to obtain a deeper insight into the metabolism of choline in *Aspergillus niger* of Tieghem.

2. DISSIMILATION OF CHOLINE

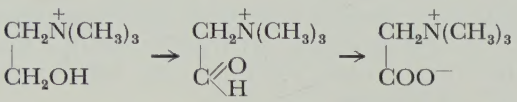
The dissimilation of choline in the organism can proceed in two different ways:

a. the breakdown of choline to trimethylamine; in some cases trimethylamine is still oxidised to trimethylaminoxide.



$$\begin{matrix}CH_2\overset{+}{N}(CH_3)_3\\ |\\ CH_2OH\end{matrix} \rightarrow N(CH_3)_3 \rightarrow \begin{matrix}\overset{+}{N}-(CH_3)_3\\ |\\ O^-\end{matrix}$$

b. Oxidation of choline via betaine-aldehyde to betaine.



$$\begin{matrix}CH_2\overset{+}{N}(CH_3)_3\\ |\\ CH_2OH\end{matrix} \rightarrow \begin{matrix}CH_2\overset{+}{N}(CH_3)_3\\ |\\ C\begin{smallmatrix}/\!\!/O\\ \diagdown H\end{smallmatrix}\end{matrix} \rightarrow \begin{matrix}CH_2\overset{+}{N}(CH_3)_3\\ |\\ COO^-\end{matrix}$$

2. 1 *The degradation of choline to trimethylamine*

The degradation of choline to trimethylamine has been described by Cromwell (39) in leaves of *Chenopodium vulvaria L.* Dyer and Wood (49) discovered that in certain *Enterobacteriaceae* choline and acetylcholine break down into trimethylamine; betaine was not transformed. Cohen and his collaborators (36) observed a degradation of choline to trimethylamine, ethanolamine, ammonia and ethyleneglycol in some species of *Clostridium.* During their investigations on the creatine-synthesis with minced muscle of caviae Barrenscheen and Pantlischko (5) came to the conclusion that besides the oxidation of choline to betaine another way exists whereby choline is degradated; the end-product in this case would be trimethylaminoxide.

2. 2 *Oxidation of choline to betaine*

The oxidation of choline to betaine via betaine-aldehyde has been investigated by Bernheim and Bernheim (14, 15). After adding choline to an extract of rat-liver they determined the quantity of oxygen absorbed at different pH; it seemed that at pH 6.7 one and at pH 7.8 two atoms of oxygen per mol choline were used. In the faintly acid mixture the presence of betaine-aldehyde could be proved as a bisulphite-compound. In contrast to the alcaline the acid solution showed reducing properties. The presence of a choline-dehydrogenase which can oxidise choline via betaine-aldehyde to betaine was established.

After an elementary analysis of the reineckate compound of the oxidation product of choline Mann and Quastel (96) too came to the conclusion that choline is transformed enzymatically to betaine-aldehyde by rat-liver extract. In a subsequent publication they demonstrated that the enzymesystem was composed of two components viz.: choline-dehydrogenase which was not inhibited by small quantities of cyanide and of a cytochrome- and indophenol-oxydase. In anaerobic conditions the oxidation of choline could also be arrived at, provided a suitable hydrogen-acceptor (sodium-ferricyanide) was added. The fact that choline-dehydrogenase does not oxidise alcohol (Bernheim c.s.), indicates that the alcohol-group does not obstruct the enzyme: in this respect the group-NR_3 is of importance because choline-dehydrogenase is inhibited by NH_4^+, $NH(CH_3)_3^+$ and betaine.

The presence of a choline-oxidising enzyme is not restricted to the animal organism. According to a recent publication by Cromwell and Rennie (40) this enzyme is also present in the roots of *Beta vulgaris;* the oxidation is accelerated in vitro by cytochrome *c*; the cyanide ion tends to retard.

The oxidation of choline is now of great importance. On the one hand the methylgroups of betaine are transferred by a transmethylation reaction to other compounds (2.2.1), on the other hand they can be oxidised to CO_2 via formate (2.3.).

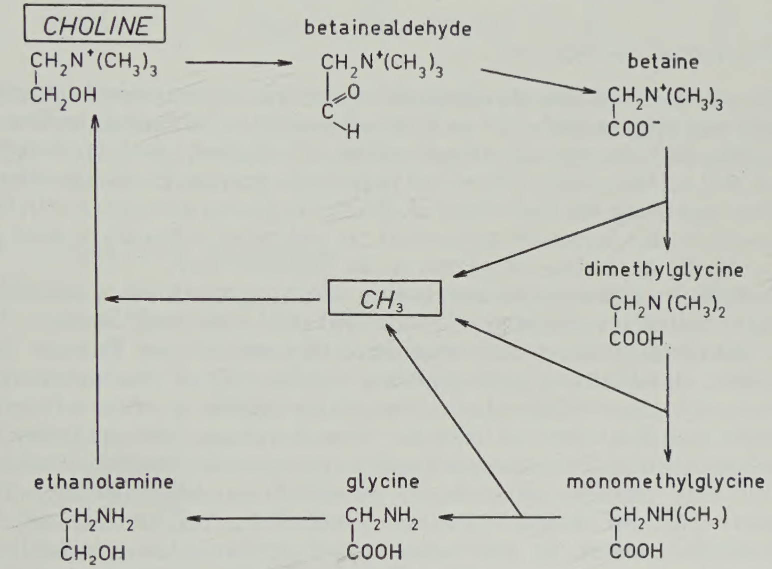
2.2.1 *The indirect methylation action of choline*

The experiments of Rose (109) and du Vigneaud (140) had shown that the amino acid methionine which is indispensable to rats could be replaced by homocysteine plus choline in their diet; Borsook and Dubnoff (23) have demonstrated a synthesis of methionine from homocysteine and choline with homogenates of rat-liver, whereas Simmonds and others (119) with the aid of isotopes proved the transfer of a methylgroup of choline during the methionine-synthesis. To obtain a closer insight into the procedure of the reaction of homocysteine + choline → methionine, it proved necessary to investigate whether choline had a direct methylating action whether it is first transformed into another compound.

The experiments of Borsook and Dubnoff (23) gave rise to the conjecture that choline was not directly involved in the transmethylation: under aerobic and anaerobic conditions the methylation of homocysteine in rat-liver homogenates by betaine proved to be quicker than by choline; a closer investigation moreover (42) showed that the methylation of homocysteine by choline in liver homogenates of different animals correlated with the contents of choline-oxidase of the livers of these animals. Thus the livers of caviae and rabbits contained no choline-oxidase, those of mice, chickens and rats did. The methionine-synthesis from choline and homocysteine by liver homogenates of caviae and rabbits was slight compared to those by liverhomogenates of mice, chickens and rats under similar conditions.

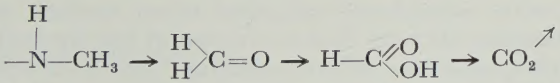
Muntz (106) provided a more direct proof of the methylating action of choline linked to an oxidation; he incubated a rat-liver homogenate under anaerobic conditions with homocysteine and choline containing N^{15}. With the aid of the "carrier-technique" it was found that after incubation the dimethylethanolamine present contained only slight quantities of N^{15} and the dimethylglycine on the contrary considerable quantities of N^{15}. According to these experiments the transfer of a methylgroup of choline apparently requires in the first place the oxidation of the alcoholgroup of choline to the carboxylgroup of betaine.

The procedure of the transfer of a methylgroup of choline as formulated by Muntz (106) corresponds with the earlier experiments of Stetten (128, 129). Stetten fed rats betaine containing N^{15} and discovered that after 3 days most of the N^{15} appeared in glycine, slightly less in ethanolamine whereas choline contained the smallest quantity. du Vigneaud and collaborators later repeated these experiments with betaine which besides N^{15} contained deuterium in the methylgroups; they discovered betaine to be an extremely active methyldonor. Most of the N^{15} was found in glycine, whereas a small quantity was also present in choline (141). The following scheme summarizes these reactions:



2.3. *Oxidation of the labile methylgroup.*

The action of the labile methylgroup of compounds as choline, betaine and methionine is not only limited to a transfer to a methylacceptor. The experiments of Mackenzie (93) proved that the rat can oxidise a labile methylgroup of compounds as methionine and choline to CO_2. The oxidation of the methylgroup of sarcosine (monomethylglycine) proceeded via formaldehyde and formic acid.


$$\overset{\displaystyle H}{\underset{|}{}}\!\!\!\!-N-CH_3 \rightarrow \begin{matrix}H\\H\end{matrix}\!\!>C{=}O \rightarrow H-C\begin{matrix}\nearrow\!\!O\\ \searrow OH\end{matrix} \rightarrow CO_2\nearrow$$

Ferger and du Vigneaud (53) have investigated the rate of oxidation of various compounds with labile methylgroups; the process of oxidation to CO_2 of the methylgroups of choline appeared to be much slower than that of betaine, dimethylthetine and dimethylpropiothetine.

The more rapid oxidation of the methylgroups of the last three compounds with respect to those of choline indicates a greater reactivity of the methylgroups; this coincides with the results of the experiments of MUNTZ which has already been mentioned before (106) on the indirectly methylating action of choline via betaine. Yet another indication of a link between the reactivity and the oxidability of the methylgroups became apparent from the experiments of MACKENZIE and DU VIGNEAUD (94) who discovered that rats do not produce radioactive CO_2 from creatine and creatinine which contain methylgroups with C^{14}; formerly DU VIGNEAUD and collaborators (138) had shown that the methylgroups of creatine and creatinine do not take part in the transmethylating reactions.

3. SYNTHESIS OF CHOLINE

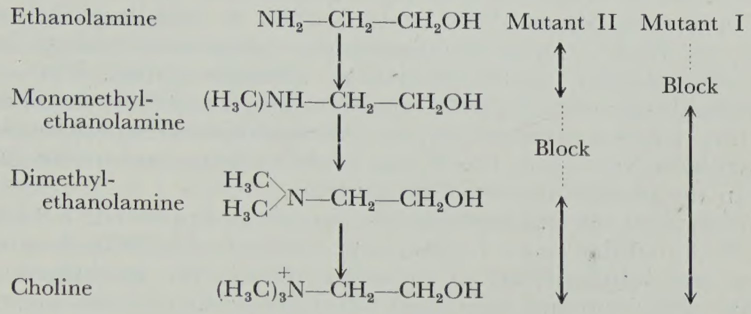
The research on the metabolism of choline in animals with compounds not tagged with C^{14} or D was founded on the observations of BEST (16, 17) on the lipotropic effect of choline, and on those of JUKES (69) on the ability of choline to prevent perosis. Such experimental data regarding the deficiency of choline in plants were not available; HOROWITZ (63) however succeeded in indirectly obtaining data on the synthesis of choline in plants in an indirect way.

Neurospora crassa synthesizes choline during growth on a minimum nutritive-solution consisting of carbohydrates, salts and biotine.

By using ultra-violet ray treatment HOROWITZ and BEADLE (64) succeeded in obtaining a choline-less mutant (I) of *Neurospora crassa* which only grew when choline was also added to this minimum nutritive-solution. At a certain concentration the quantity of mycelium produced was proportional to the quantity of choline added; in this way choline could be determined microbiologically. This mutant was no longer able to synthesize choline out of the compounds present in the minimum nutritive-solution. Addition of ethanolamine to the nutritive-solution did not produce growth; the organism was apparently unable to synthesize choline from this compound. JUKES and DORNBUSH (71) proved however that mutant I was also able to grow if dimethylethanolamine was added to the nutritive-solution. We have to conclude therefore that mutant I is able to produce choline from dimethylethanolamine. The activity of this compound calculated per mol equalled that of choline.

Later HOROWITZ (63) acquired another mutant (II), which after adding choline showed a considerable increase in growth although growth was never completely retarded when choline was lacking. Closer investigation showed that a compound was formed by mutant II on a nutritive-solution without choline, which increased growth of mutant I; this compound proved to be monomethylethanolamine.

From this it was concluded that with mutant I formation of choline from ethanolamine was completely blocked at the first step (ethanolamine -> monomethylethanolamine) whereas with mutant II the step of monomethyl -> dimethylethanolamine was retarded. In view of this it is probable that synthesis of choline in *Neurospora crassa* proceeds by methylation of ethanolamine into choline step by step.



With the aid of tracers these indirectly obtained results have been proved directly in animals. By feeding rats ethanolamine labelled with N^{15}, this was found to be present in the choline and phospholipoids of the tissues (STETTEN, 128, 129).

3.1. *Origin of the methylgroups*

When it became probable from the experiments of HOROWITZ (63, 64) and STETTEN (128, 129) that the synthesis of choline in plants and animals can be the result of methylation of ethanolamine, the question of the origin of the methylgroups immediately arose. In view of certain publications of which we will give a more detailed account later, it appears that two kinds of compounds can function as a methyldonor viz. on the one hand the compounds which comprise a biologically labile methylgroup (methionine, betaine, dimethylthetine and dimethylpropiothetine); on the other hand formate which can also function as a methyldonor according to recent investigations with tracers (1, 2, 13, 32, 99, 117, 118).

For a summary of the reactions which occur at the synthesis of choline we refer to the scheme on pag. 172; on account of a great deal of publications in connection with this, a certain curtailment proved necessary. Detailed summarizing articles regarding the metabolism of compounds with biologically labile methylgroups have been published by LUCAS and BEST (89), JUKES (70), DU VIGNEAUD (135) and BACH (4). In this summary we have especially laid stress on the metabolism of choline.

3.1.1. *Transmethylationreactions.*

During the investigation on methionine-synthesis in rats it was found that choline can yield a methylgroup to homocysteine; this reaction can be reversed so that methionine can also yield a methylgroup at the synthesis of choline. DU VIGNEAUD c.s. (136, 137) observed that after administering rats for 3 weeks methionine with 3 deuterium atoms in the methylgroup, 57 % of the deuterium could be recovered in the methylgroups of choline; after 94 days this became 88,6 %. This transfer of a methylgroup of methionine can occur not only in rats but also in human beings (120), in birds (26), in rabbits (115) and in dogs (30, 31, 77). Dimethylethanolamine, monomethylethanolamine and ethanolamine could function as methylacceptors. The earlier mentioned experiments of STETTEN (128, 129) proved that ethanolamine can function as a precursor in rats. After administering ethanolamine tagged with N^{15} to rats, this N was found to be present in the choline and in the phospholipoids of the tissues.

In the plant the transmethylation has also been proved: KIRKWOOD (79, 99) found that when l-methionine labelled with C^{14} in the methylgroup was administered to sprouting barley, the methylgroups of choline and hordenine contained C^{14}. Choline-methyl was not transferred to methionine in the barley plant.

CHALLENGER c.s. (32) showed in *Aspergillus niger* transmethylation by dl-methionine; they found that the methylation by this compound is about 20 times larger than by choline or betaine. Contrary to the rat it seems that in both plants the transmethylation of methionine to choline is irreversible.

As yet very little is known about the reaction-mechanism which takes place at the transfer of the methylgroup of methionine; literature on this subject indicates two ways in which this transfer can take place:

A. Transfer of the methylgroup of methionine via methionine-sulfoxide [BARRENSCHEEN and VON VALYI-NAGY (8)].

For their investigations they used a mash of crushed wheat seedlings to which they added glycine and methionine. After shaking 36 to 48 hrs while passing oxygen, they isolated choline and betaine; they found after adding glycine and methionine a larger quantity of choline than without addition of these 2 compounds. Unlike to BARRENSCHEEN (8) STEENSHOLT (126) could not discover any in vitro methylation of ethanolamine by methionine in a mash of wheat seedlings.

BARRENSCHEEN and others (7) have also investigated the methylation of ethanolamine in animal material that is in macerated slices of the rat; they found that the synthesis of choline is an aerobic process, during which the methylgroup of methionine by oxidation to methionine-sulfoxide will be made labile. According to these authors ethanolamine was methylated by methionine-sulfoxide under anaerobic conditions, but not by methionine. By using an extract of rat-liver STEENSHOLT (125) was also able to acquire the in vitro methylation of ethanolamine by methionine; he found that the methylating activity of methionine-sulfoxide amounts to approximately half of that of methionine.

During a later investigation STEENSHOLT (127) compared the activities of the d- en l-shape of methionine at the synthesis of choline. He discovered that the d(+)-methionine was much more active than the l(—)-methionine.

B. Transfer of the methylgroup of methionine by ATP.

In view of their in vitro experiments with rat-liver preparations

Borsook and Dubnoff (22, 23) distinguished 2 types of transmethylation reactions *a*) methylation reactions which are dependent of O_2 (the synthesis of N-methylnicotinamide from nicotinamide and methionine) and *b*) methylation reactions which are independent of O_2 (the synthesis of methionine from homocysteine and betaine).

According to Cantoni (27, 28) the first type of methylation reaction needs an energy-rich phosphate for the transfer. Originally he supposed that phosphorylated methionine was the active agent. Further investigations showed however that the activated methionine consisted of a compound of methionine with the adenosine-part of ATP (29). This S-adenosylmethionine can function without ATP as a methyldonor.

Smith c.s. (121) discovered that thiomethyladenosine can be enzymatically split off from S-adenosylmethionine; the linkage of methionine to adenosine does therefore not necessarily lead to the transfer of a methylgroup.

Finally it should be pointed out that the synthesis of choline is supposed to belong to the aerobic reaction type which necessitates the presence of ATP.

Besides methionine some other compounds with biological labile methylgroups can function as methyldonor; du Vigneaud c.s. (141) discovered that at the synthesis of choline betaine was an extremely active methyldonor.

With regard to this type of methylation reactions Dubnoff and Borsook (44, 45) like Maw and du Vigneaud (101) link the lability of the methylgroup of betaine under anaerobic conditions with the structure of the betaine-molecule. In general, especially the compounds with an "onium" structure such as betaine and the S-analogue dimethylthetine under anaerobic conditions combine a methylating capacity with their growth-promoting properties. The reaction-mechanism of methylation by "onium" compounds as betaine is not known; further research will have to prove whether betaine is the methyldonor or whether it is first changed into other compounds.

Besides betaine the S-analogue of this compound can also function as a methyldonor at the methionine-synthesis (du Vigneaud, 105, 139); when homocysteine plus sulfobetaine (dimethylthetine) is added to the diet of a rat which lacks choline and methionine, the animal will grow normally. Maw and du Vigneaud (100, 102) have investigated some compounds related to dimethylthetine with regard to their growth-promoting action. Replacement of one methylgroup (methylethylthetine) resulted in a decrease of the growth and replacement of 2 methylgroups (diethylthetine) caused a cessation of growth. The homologue S-dimethylpropiothetine was as active as dimethylthetine; the first compound was isolated from algae by Challenger and Simpson (33). Dubnoff and Borsook (44, 45) investigated the in vitro methylation of homocystine by these S-compounds; they found that the transfer of the methylgroups of these S-compounds occur under the influence of a specific enzyme of the rat-liver. The methylating action of dimethylthetine and dimethylpropiothetine was several times larger than that of betaine. The methylating action of the S-analogue of choline, sulfocholine has also been tested; this compound however was toxic to rats [MAW and DU VIGNÉAUD (101)].

3.1.2. *Synthesis of methylgroups by the organism.*

In the introduction it has already mentioned that the methylgroup in plants and in animals are of different origin: in animals the methylgroup required for the synthesis of choline are for the greater part present in the diet. This is not the case in plants; that's why the plant must synthesize the labile methylgroups. In this connection the following experiments with animals are of special importance.

During his first fundamental investigations on the methylation of homocysteine by choline in rats on a diet lacking compounds with labile methylgroups DU VIGNEAUD (139) mentioned that now and again some animals, after first losing weight, started to grow again without the addition of choline to their diet. It was thought that this was caused by a possible synthesis of labile methylgroups by the micro-flora of the intestines. BENNETT (11, 12) later confirmed this incidental growth in some animals; after feeding them sulfasuxidine however, these animals ceased to grow. A new aspect presented itself when it became apparent that after cessation of growth due to sulfasuxidine growth would be resumed when a crude liver extract was administered. The formation of the methylgroups was probably not due to bacteria but to a vitamin factor of the liver extract.

BENNETT came to the conclusion that "There may be vitamin factors of either dietary or intestinal origin, the presence of which may enable the animal to compensate for the absence of dietary methyldonors by biosynthetic means of its own or of its intestinal bacteria."

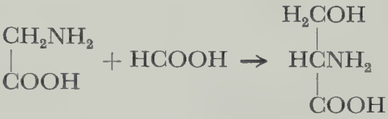
Some time afterwards the vitamin B_{12} was isolated in the liver. Later this appeared to be part of the A(nimal) P(rotein) F(actor). PATTON c.s. (107) proved, as did BIRD and others (18) that the APF-factor affected the need for methionine in chickens. Other observations regarding a relation between the metabolism of biologically labile methylgroups and the vitamin B_{12} were those of SCHAEFER (114) and GILLIS and NORRIS (55).

In 1949 BENNETT (10) discovered that vitamin B_{12} plus folic acid increased the growth of young rats in the same way as the formerly used crude liver extract. JUKES and STOKSTADT (72) found that in chickens the need of choline and methionine was reduced by vitamin B_{12}. These experiments clearly proved that labile methylgroups were synthesized under the influence of vitamine B_{12}; the question remained whether this synthesis was produced by the tissues of the rat rather than by the flora of the intestines. DU VIGNEAUD (142) in collaboration with REYNIERS of the Lobund Research Institute has solved this problem as follows: D_2O was administered to completely sterile raised rats fed on a diet containing vitamin B_{12} and folic acid. As controls non sterile animals raised on the same ration were used. After 10 days in the body fluids of the sterile animals 3.3 % of the total quantity deuterium was found in the methylgroups of choline, after 23 days this amounted 6.4 %. The quantity of deuterium present in the methylgroups of choline was slightly more in non sterile animals which indicates a possibly extra synthesis of the methylgroups by the flora of the intestines.

When it became apparent from these experiments by DU VIGNEAUD that, if vitamin B_{12} was present, a rat could synthesize biologically labile methylgroups, all interest was centered on the compounds involved in this synthesis. In this connection MACKENZIE's experiments (93) on the oxidation of the labile methylgroup via formaldehyde and formic acid are of importance (see pag. 166); besides the methylgroup can be oxidised to CO_2. The function of formic-acid in the synthesis of the labile methylgroup can be gathered from the following experiments.

In 1948 SAKAMI (110) discovered that rats were able to form serine out of formic acid and glycine, whereas a year later it appeared that the methylgroup of choline labelled with C^{14} reappeared as the β-carbon atom of serine (111)

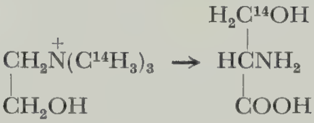
(*a*) glycine



$$\begin{array}{l} CH_2NH_2 \\ | \\ COOH \end{array} + HCOOH \rightarrow \begin{array}{l} H_2COH \\ | \\ HCNH_2 \\ | \\ COOH \end{array}$$

serine

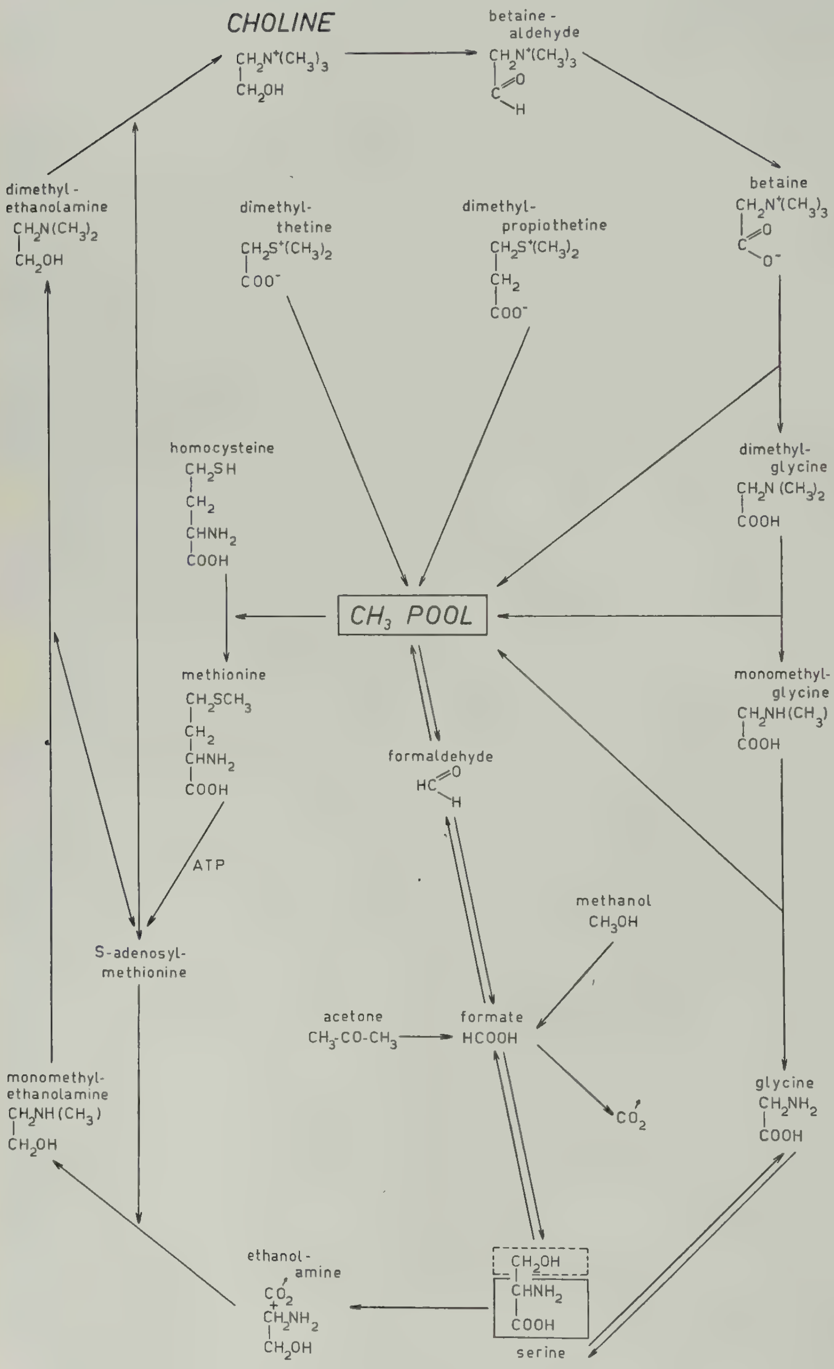
(*b*) choline



$$\begin{array}{l} CH_2\overset{+}{N}(C^{14}H_3)_3 \\ | \\ CH_2OH \end{array} \rightarrow \begin{array}{l} H_2C^{14}OH \\ | \\ HCNH_2 \\ | \\ COOH \end{array}$$

serine

In view of these reactions it seemed probable that the C atom of the methylgroup of choline appeared in serine (reaction b) via formic acid. On closer investigation the reaction $—C^{14}H_3 \rightarrow HC^{14}OOH \rightarrow H_2C^{14}OH\text{-}CHNH_2\text{-}COOH$ proved to be reversible; thus SAKAMI and WELCH (113), SIEKEVITZ and GREENBERG (117, 118) and BERG (13) discovered that the carbon atom of formic acid reappeared in the methylgroups of methionine. These experiments were extented by ARNSTEIN (1, 2); JOHNSSON and MOSHER (68) and WEISSBACH (146), who used C^{14} labelled aminoacids; thus the α-carbon atom of glycine and the β-C atom of serine were recovered in the methylgroups of choline. If, for example, serine with a β-C^{14} atom $C^{14}H_2OH\text{-}CHNH_2\text{-}COOH$ was fed to a young rat the methylgroups as well as the ethanolamine-part of the choline in the liver contained C^{14}; apparently serine does not only function as a methyldonor but is after decarboxylation a methylacceptor too. Besides formic acid (13, 113, 117, 118), glycine (2) and serine (2) are compounds as acetone (108), methanol and formaldehyde (138, 139) active in the synthesis of biologically labile methylgroups. The above mentioned reactions have been comprised in the following scheme:


CHOLINE
$CH_2N^+(CH_3)_3$
CH_2OH
betaine-aldehyde
$CH_2N^+(CH_3)_3$
$C{=}O$
H
betaine
$CH_2N^+(CH_3)_3$
$C{=}O$
O^-
dimethyl-ethanolamine
$CH_2N(CH_3)_2$
CH_2OH
dimethyl-thetine
$CH_2S^+(CH_3)_2$
COO^-
dimethyl-propiothetine
$CH_2S^+(CH_3)_2$
CH_2
COO^-
dimethyl-glycine
$CH_2N(CH_3)_2$
COOH
homocysteine
CH_2SH
CH_2
$CHNH_2$
COOH
CH_3 POOL
monomethyl-glycine
$CH_2NH(CH_3)$
COOH
methionine
CH_2SCH_3
CH_2
$CHNH_2$
COOH
formaldehyde
$HC{=}O$
H
ATP
methanol
CH_3OH
S-adenosyl-methionine
acetone
$CH_3\text{-}CO\text{-}CH_3$
formate
HCOOH
CO_2
monomethyl-ethanolamine
$CH_2NH(CH_3)$
CH_2OH
glycine
CH_2NH_2
COOH
ethanol-amine
CO_2
+
CH_2NH_2
CH_2OH
CH_2OH
$CHNH_2$
COOH
serine

From the above it appears therefore that besides the labile methylgroups, which are at the disposal of the animal in its diet, a synthesis of methylgroups is possible under the influence of the vitamin B_{12}, whereby formic acid is an important precursor. Under normal conditions in animals the diet is the most important source of the labile methylgroups; the synthesized methylgroups are sometimes the cause of the non-appearence of certain deficiency phenomena such as the absence of growth when methionine is lacking.

The plant has no substances with labile methylgroups at his disposal. That is why the plant is practically always dependent on the synthesis of the labile methylgroups from substances like formaldehyde and formate. Kirkwood c.s. (99) and Challenger (32) produced the experimental proof of this supposition; they isolated compounds with C^{14} in the methylgroups after feeding $NaC^{14}OOH$ to sprouting barley resp. *Aspergillus niger.*

A comparison of formate and methionine as precursors of biologically labile methylgroups showed that the latter was much more efficient. This supports the idea that formate serves as a precursor of methionine-methyl.

With these results in mind we formulated a central "CH_3 pool" as shown in the scheme above from which a methylgroup can be transferred via methionine and S-adenosyl-methionine to the precursors of choline (ethanolamine, mono- and di-methylethanolamine).

4. Incorporation of choline

To be able to acquire a more comprehensive idea of the metabolism of choline a survey of the synthesis and hydrolysis of the derivatives of choline should follow on the discussion of the dissimilation and synthesis of free choline. The choline-containing compounds of the organism can be divided in the phosphatides lecithine and sphingomyeline, in sinapine present in some plants and in acetylcholine found chiefly in animals.

4.1. *The phosphorus containing compounds of choline*

Biochemically and physiologically, the phosphatides appear to be involved in a multiplicity of functions of the greatest importance, about which knowledge is scanty. The phosphatides are active metabolic components of many organs and tissues, particularly the gastrointestinal tract, the liver and the blood. Very little is known about the course of phosphatide metabolism in plants, firstly because of the difficulty of the work involved and secondly because phosphatides are, for the most part, rather minor constituents of the members of the plants.

Ducet (46) proved that during the vegetative growth of soy a synthesis of phosphatides takes place at the expense of watersoluble choline. During the ripening of the seed a dissimilation of the phosphatides sets in whilst choline is liberated.

The hydrolysis of lecithine can proceed in various ways; there are four points of attachment (ester linkages), which should prove susceptible to enzymatic attack. These comprise the first and second fatty acid linkage, the phosphoric acid-glycerol linkage and the aminoalcohol-phosphoric acid linkage. Accordingly, the existence of lecithinases corresponding to each of the ester linkages of lecithin was postulated. These are defined as follows:

Lecithinase A: An enzyme which removes one fatty acid from lecithin.
,, B: An enzyme which removes the remaining acid from lecithin.
,, C: An enzyme with cholinephosphatase activity capable of splitting choline from lecithin.
,, D: An enzyme with glycerophosphatase activity which separates lecithin at its ester linkage between glycerol and phosphoric acid.

An example of the occurrence of the lecithinase C activity in the higher plant has been shown in the beautiful investigations of Hanahan and Chaikoff (59, 60, 61). They found that the carrot phosphatides, which could be extracted with alcohol-ether mixtures from raw carrots were characterized by a low nitrogen content and by a virtual absence of choline. The same extraction when applied to steam-treated carrots, provided a more conventional phosphatide with a molar ration of 0.89 of nitrogen-phosphorus. The product extracted from raw carrots appeared to be similar to the phosphatidic acids; and it seemed logical, in view of the effect of prior treatment with steam, to postulate the presence of an enzyme which split the nitrogenous base from the phosphatide during the isolation process. This was substantiated in further work by Hanahan and Chaikoff (60), who were able to isolate from carrots an enzyme extract, which possessed no choline-esterase activity, but which split the ester linkage between the nitrogenous base and the phosphoric acid grouping in phosphatides obtained from carrots and soybeans. The enzyme exerted its maximum activity at a pH range of 5.2 to 5.9; it showed a high degree of heat stability since it was not completely inactivated when exposed to a temperature of 95° C for 15 minutes.

A similar enzyme was shown to be present in cabbage leaves (61) and, as in the case of carrots, a normal phosphatide could be isolated only after the cabbage leaves had been subjected to steam treatment. From the fresh leaves, phosphatidic acids were isolated. The enzyme in cabbage leaves exhibited its maximum activity in the same pH range as the carrot enzyme and it was quite thermostable too.

The term lecithinase A was applied by Contardi and Ercoli (37) to the enzyme which removes one fatty acid from lecithin; lecithinase A was originally found in cobra venom, where it was detected because it reacted with lecithin to yield a hemolytic substance. The enzyme was also found in the pancreas, dried heart, liver, spleen, muscle, brain, suprarenals thymus and prostate. The fresh organs contained only lecithin, indicating that the lecithinase A was in some manner held in check in the fresh organ by other enzymes or protective substances.

These materials were apparently inactivated during the drying process, leaving the lecithinase A free to act on the lecithin. Lecithinase A is also found in certain substances of vegetable origin, although its occurrence is apparently not as wide as in the animal kingdom. IWATA (66) found it in polished rice, and FRANCIOLI (54) obtained evidence for its occurrence in a fungus *Lycoperdon giganteum.*

The lecithinase B removes the remaining acid from lysolecithin; the enzyme appears to be present in higher fungi, in most animal organs and in blood. It is also found in some higher plants like soja (46), *Lolium perenne* (see chapter III) and leaves of potatoes. (46). The pH for the optimum activity of lecithinase B was determined by CONTARDI and ERCOLI (37) by noting the value at which the hemolytic activity of lysolecithin disappeared most rapidly. The value proved to be 3.5 which is quite different from the neutral value found to be the optimum for lecithinase A.

The enzyme which hydrolysed lecithin at the glycerol and phosphoric acid ester linkage may be referred to as lecithinase D. It was found by MACFARLANE and KNIGHT (95) in *Clostridium welchii;* the enzyme appears to be a component of the bacterial toxin elaborated by this organism.

The action of the lecithinases is summarized in the chart on page 176:

Besides lecithin sphingomyeline also occurs in the phosphatides of the brains and spinal cord; LEVENE (84) was the first to obtain relatively pure sphingomyelin and to report that the hydrolysis products consisted of phosphoric acid, choline, sphingosine and fatty acids (chiefly lignoceric acid).

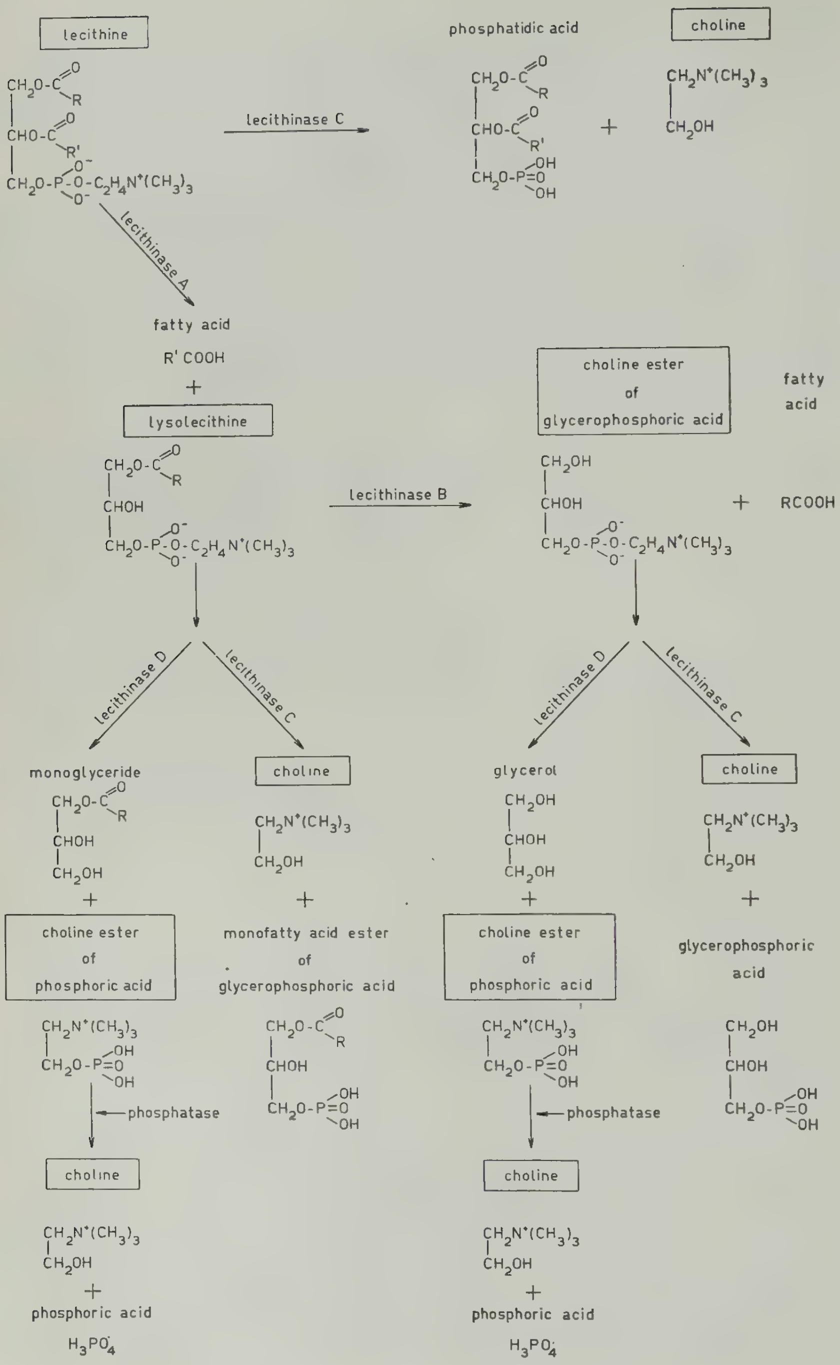
Very little is known about the biochemistry of sphingomyelin, save that in Niemann-Piek's disease the sphingomyelin content of the liver and the spleen increases greatly at the expense of the brain cerebrosides. Thus it appears that sphingomyelin plays a role in lipide metabolism (82, 133).

4.2. *Sinapine*

Sinapine is isolated from mustard (122); it occurs as the glycoside sinalbine which is composed of sinapine, glucose and p-oxybenzyl-mustardoil. Sinapine is the ester of choline and 3,5 dimethoxy-4-oxycinnamicacid (sinapine-acid); practically nothing is known about the metabolism of this compound.

4.3. *Acetylcholine*

Besides the role in the transmethylation reaction and in the lipide metabolism choline has still another function which is not yet too clearly understood. This involves the acetylation of choline in the organism to yield acetylcholine. The acetylation and deacetylation of choline seem to proceed at will in the animal, depending apparently on whether the blood pressure level is in need of lowering. DALE (41) showed that acetylcholine stimulates the parasympathic nerves as well as the sympathic ganglia and probably the autonomic ganglia. It seems to be the chemical agent involved in the transmission of nerve

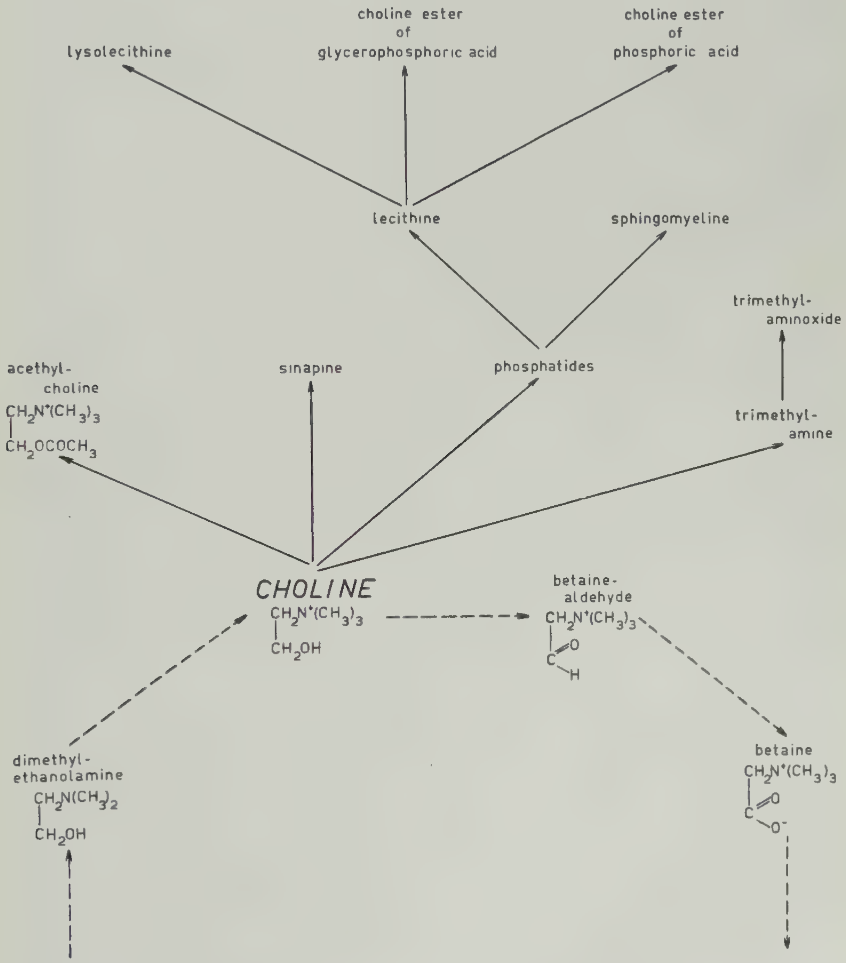

lecithine
lecithinase C
phosphatidic acid
+
choline
lecithinase A
fatty acid
R' COOH
+
lysolecithine
lecithinase B
choline ester of glycerophosphoric acid
fatty acid
+
RCOOH
lecithinase D
lecithinase C
lecithinase D
lecithinase C
monoglyceride
+
choline ester of phosphoric acid
phosphatase
choline
+
phosphoric acid
H3PO4
choline
+
monofatty acid ester of glycerophosphoric acid
glycerol
+
choline ester of phosphoric acid
phosphatase
choline
+
phosphoric acid
H3PO4
choline
+
glycerophosphoric acid

impulses to muscles. The enzyme cholineesterase is present in many tissues and readily hydrolyzes the acetylcholine, thereby controlling its action. A high concentration of the esterase is found at or near the surface of the nerve cell.

With regard to plants acetylcholine has been found in ergot (52) and in shepherd's purse (24), whereas it is also present in the squeezed juice of beets, turnips and gherkins. The presence of acetylcholine in the juice of sour gherkins and in the squeezed juice of silage would be caused by *Bacillus acetylcholini* (58, 76, 104).

5 SUMMARY

Present knowledge regarding the metabolism of choline is summarized in the following chart:



Chapter II

DETERMINATION OF CHOLINE AND BETAINE IN VEGETABLE MATERIAL

1. Introduction

Choline may be found in plants either as free or bound choline; of the latter 2 forms are known viz. watersoluble bound and water insoluble bound or lipoid choline. Schmidt and collaborators (116) proved that watersoluble bound choline of the pancreas equals the choline ester of glycerophosphoric acid. On the analogy thereof one assumes that the watersoluble bound choline of the plant is the same compound; this has however not yet been proved. The water insoluble bound choline of the plant occurs as lecithin. Regarding the study of the metabolism of choline in plants it is desirable to be able to determine the quantities of free, watersoluble bound and of water insoluble bound choline, besides the quantity of total choline. Moreover, during the experiments on the dissimilation of choline by *Aspergillus niger*, it proved necessary to determine betaine also. This chapter will successively deal with the literature regarding the determination of free, watersoluble, waterinsoluble and total choline, the determination of betaine, after which a more detailed account will be given about the procedures of these determinations.

2. The most important methods of determining choline

Literature dealing with the determination of choline has been excellently summarized by Guggenheim (57), Lucas and Best (89); we shall only confine ourselves at this stage to a short discussion on the various methods, at the same time referring to some later publications on this subject.

Choline can be determined either physiologically, microbiologically or chemically.

The oldest and most sensitive method is the physiological one [Hunt and de Taveau (65)], which depends on the measurement of the contraction of a smooth muscle caused by acetylcholine. The activity of choline is far smaller than that of acetylcholine; the determination can be interrupted by alcohol, Ca, K, lecithin, traces of protein and cholesterol. By this method 0,1 γ of choline can even be determined.

For the microbiological determination of choline Horowitz and Beadle (64) use a "choline-less" mutant of *Neurospora crassa*. This they acquired from the wild form of this mould by radiation with ultraviolet light. On a nutritive-solution of sugars, salts and biotine the mutant showed very little growth unless small quantities of choline were added. At a certain degree of concentration the quantity of mycelium formed was proportional to the quantity of choline added to the nutritive-solution.

Several amino acids and water-soluble vitamins have been examined for their growth-promoting activity for this mutant; it appeared that only methionine was able to promote growth slightly (approx. 1/500th part of the activity of choline); approx. 10 γ choline can still be determined by this method.

The methods to determine choline chemically can be divided into:

a) determination of choline as trimethylamine. This method depends on the formation of trimethylamine out of choline by oxidation with potassium permanganate under alcaline conditions (87, 88, 131) or by boiling in concentrated lye (80, 81); after titration of the trimethylamine the quantity of choline can be calculated. The smallest quantity of choline to be determined by these methods approximates 1 mg; in that case however the average discrepancy is very large (approx 30 %) whereas quantities of 30–50 mg choline are necessary for a rather accurate estimation. To determine the percentage of choline present in moulds these methods are not suitable.

b) determination of choline as choline-reineckate. BEATTIE (9) uses this precipitate to determine choline colorimetrically; later JACOBI, BAUMANN and MEEK (67), ENGEL (50) and GLICK (56) introduced some modifications with the aim to increase the specificity of the determination (50, 67) and to counteract the loss of choline-reineckate caused by the washing of the precipitate (56). With this method choline is precipitated as choline-reineckate, the precipitate is sucked off, washed and after dissolving in acetone the intensity of the rose coloured solution is measured at 525 mμ. Besides this maximum of absorption in the visible part of the spectrum a larger maximum of absorption exists at 327 mμ according to WINZLER and MESERVE (148) After measuring in the ultra-violet zone 50 μg of choline-chloride can still be determined, whereas the smallest quantity at 525 mμ is approx. 1 mg. The determination in biological material is not possible without pretreatment as besides choline other compounds may be precipitated. (48, 73, 130)

c) determination of choline as enneaiodide. The precipitation of choline with a solution of potassiumtriiodide has been described by STANEK (123); later several investigators (46, 47, 51, 108) used this reagens for the determination of choline. With these methods choline is precipitated with a surplus of the potassiumtriiodide under the formation of a greenish black cristalline precipitate of choline-enneaiodine. This is easily recognizable under a microscope by its characteristic cristalline shape of narrow obligue prisma's. After leaving some time in the refrigerator it is centrifuged and the above fluid is drained off. After rinsing some times with icewater the quantity I bound by choline is determined by titration with sodiumthiosulphate. The percentage of choline can be calculated from the formula of choline-enneaiodine $[I_8.\ IN(CH_3)_3.CH_2CH_2OH]$: per mol choline 8 atoms of I are titrated. With DUCET's method 0,5 mg choline-chloride can still be determined (46, 47); ERICKSON and collaborators (51) have increased the sensitivity of the determination 6 times by oxidising the I in the choline-enneaiodide to iodate.

d). The determination of choline with paperchromatograms. To procure the separation of the N-containing substances of the lipoid fraction from animal material LEVINE and CHARGAFF (85, 86) used the paperchromatographic method; after hydrolysis of the lipoid fraction and removal of the fatty acids serine, ethanolamine and choline are separated paperchromatographically by developing with a mixture of butanol, diethyleneglycol and water. The place of choline on the paperchromatogram can be found after spraying with a solution of phosphomolybdic acid; the surplus of phosphomolybdic acid is removed and the choline-phosphomolybdate is reduced with stannous chloride after which a bluish spot appears. According to BRANTE (25) choline can also be located on the chromatogram by holding the paper in I-fumes; in accordance with this preliminary statement, the position of the amines, ethanolamines and betains can be located on the paperchromatogram. For the quantitative determination of the choline LEVINE and CHARGAFF (86) measured the surface of the blue choline spot on the chromatogram; between the logarithm of the choline concentration and the surface of the choline spot they found a linear relationship. The concentration of the choline solutions should be approximate 0.05–0,75 %. The exactitude of this method is limited by the accuracy with which the surface of the choline-spot can be measured.

3. DESCRIPTION OF THE METHODS USED

a Determination of choline as reineckate.

Choline is precipitated from its solution by adding 6 ml of a freshly prepared 2% solution of ammonium-reineckate in methanol. After standing for 4 h in a refrigerator the formed choline-reineckate is sucked off on a fritted glass (SCHOTT G_3) filter and rinsed 3 × with 2.5 ml amylalcohol. Afterwards the choline-reineckate is dissolved in a known volume of acetone and the intensity of the rose coloured solution measured in the stufenphotometer with a S 53 filter.

The amount of choline is calculated by using a calibration-curve. According to ENGEL (50) the extinction is not proportional to the amount of choline-chloride; other investigators (56, 67, 98, 147, 148) found however that Beer's law was applicable, although the values found for the specific extinction-coefficients were not the same. Proceeding from choline-reineckate (prepared from pure choline-chloride by precipitating with ammonium-reineckate and recristallizing from a watery acetone-solution) we calculated the specific extinction-coefficients and found the values mentioned in table I.

The specific extinction-coefficient found by us shows the greatest equivalence with that found by WILLSTAEDT c.s. (147) who also used a stufenphotometer.

b Determination of choline as enneaiodide.

To determine choline as enneaiodide 5 ml of a solution is transferred into a centrifuge-tube with a long drawn-out point, after which 1,5 ml potassiumtriiodide-solution (160 g I_2 and 240 g KI per liter) is added. Upon standing 4 h in a refrigerator it is centrifuged for 3 minutes and the surplus reagens drawn off over the precipitate with a double-bent capillary tube which at one end is fitted with a quick filtering filterpaper. The diameter of the capillary tube is such that the end connected with the small filterpaper can be brought within close range of the precipitate in the extreme point of the centrifuge-tube, without however touching the precipitate. The inside of the centrifuge-tube is again rinsed with ice-water and care should be taken that no air is sucked through in order to prevent as much as possible the dissolution of the choline-enneaiodide. After washing, traces of enneaiodide which have been sucked up, are dissolved in alcohol from the filter and squirted into the centrifuge-tube. Upon solution of the choline-precipitate in more alcohol the quantity of bound I is titrated with 0,05 n sodiumthiosulphate. The calculation is based on 8 atoms I per mol choline-chloride i.e. 1 ml 0,050 N potassiumthiosulphate equals 0,875 mg choline-chloride.

TABLE 1

Determination of the specific extinction-coefficient

mg choline-chloride per ml	mg choline-reineckate per ml	k 1 cm	Specific extinction-coefficient	
			calculated	Literature-values
1,12	3,388	0,806	0,72	0,86 (143)
0,84	2,541	0,596	0,71	0,80 (54, 95)
0,56	1,694	0,400	0,71	0,736 (142)
0,42	1,271	0,302	0,72	0,66 (65)
0,21	0,635	0,154	0,73	
0,14	0,424	0,103	0,74	
		Average	0,72	

4. THE PREPARATION OF BIOLOGICAL MATERIALS BEFORE THE DETERMINATION OF TOTAL CHOLINE

Before determining total choline an extraction and hydrolysis should first take place.

The extraction of free and bound choline from biological materials has been investigated by Engel (50). He acquired his highest results after 24 hours of extraction with methanol. He then saponified the residue of the methanol extract by heating to 100° for 2 hours with a saturated solution of barite; after neutralising with acetic acid and filtrating choline is determined as reineckate.

It appears however that with this method as with the modification of Glick (56) choline-reineckate is greatly polluted by the accompanying materials; after dissolving the reineckate in acetone one obtained a cloudy solution which did not clear after filtration. The method was therefore altered. By clearing with lead-acetate after saponifying with barite a precipitate of fine crystalline choline-reineckate was obtained which was not polluted by other materials and when dissolved in acetone gave a completely clear solution. The determination of total choline proceeded as follows:

1 g dried and powdered mycelium of *Aspergillus niger* is extracted for 20 h in a sohxlet with absolute methanol. Upon evaporation of the methanol the residue is saponified with a 30 ml of a saturated barite solution on heating for 2 h at 100°; after cooling the pH is brought at 4 with acetic acid and cleared by adding ½ g lead-acetate dissolved in a little water. The precipitate is then sucked off over a asbestos filter and rinsed; surplus lead in the filtrates is removed with 10 ml of a 10% solution of secondary sodiumphosphate. Afterwards the pH is put at 8 with lye and the precipitate of lead- and bariumphosphate is sucked off over an asbestos-filter. After washing the precipitate the combined filtrates are evaporated and finally filtrated; its volume then amounts to approx. 40 ml. With this solution

choline can only be determined as a reineckate; not as an enneaiodide as the presence of disturbing materials prevents this.

A completely different method was used by DUCET (46); it is based on the fact that choline and betaine in comparison with various other organic N-compounds are much more stable with regard to boiling mineral acids. The material is boiled with diluted nitric acid which liberates the bound choline; moreover a number of organic compounds which interfere with the determination of choline, are decomposed. A clarification then follows with calciumcarbonate and ferrisulphate acc. to LUNEAU (90).

The determination of total choline proceeds as follows:

1 g dried and pulverized mycelium of *Aspergillus niger* is refluxed 4 h with 2 ½ ml nitric-acid and 5 ml water; about ½ g of norit *) is added, the mixture neutralised with calciumcarbonate and after heating cleared with 10 ml of a 50% ferrisulphate solution. After boiling again some calciumcarbonate is added and the precipitate which consists chiefly of calciumsulphate, calciumcarbonate, ferrihydroxide and norit is drawn off. The precipitate resuspended in water, again boiled and drawn off after which the procedure is repeated another time. After evaporation of the filtrates on a waterbath the concentrate is boiled with some norit, drawn off, whereafter the precipitate of norit is boiled 3 times with warm water; the filtrates are filled to 25 ml. In an aliquot part of the solution choline can be determined as reineckate or as enneaiodide.

5. DETERMINATION OF THE DIFFERENT CHOLINE-FRACTIONS

As far as we know KLEIN and LINSER (80, 81) were the first to try to determine the choline-fractions in plants; to accomplish this they made use of the slight solubility of free choline in dry ether. According to this method an extract of alcohol is shaken with ether and water; after separation part of the ether-layer is evaporated and the lipoid bound choline determined. One of the objections to this method is that a correction must always be made for the free choline dissolved in the moist ether. This difficulty is avoided by DUCET's method (46); the material is boiled with water and the resulting suspension is cleared with calciumcarbonate and ferrisulphate.

The filtrate contains free and watersoluble bound choline. In this filtrate free choline can be directly determined; upon hydrolysis with diluted nitric acid the total amount of watersoluble choline can be found. Moreover it is possible after adsorption to silicagel on determine the watersoluble bound choline directly.

Lipoid bound choline can be determined by eliminating the surplus calciumcarbonate and ferrihydroxide from the clearing-precipitate, treating the residue with nitric acid or sulphuric acid and determining the freed choline. The separation was made as follows:

1 g dried and powdered mycelium and a little calciumcarbonate are boiled with water until every particle is properly moistened. This mixture is then cleared by adding 5 ml of a 50%-solution of ferrisulphate and some calciumcarbonate; after boiling a moment the solution is drawn off. The precipitate is resuspended in water and again drawn off after boiling. This procedure is once more repeated. Water soluble choline can be found in the combined filtrates whereas the lipoid bound

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*) Medicinal norit of the General Norit Co. Ltd. at Amsterdam showed a marked discoloration whilst no choline was adsorbed.

choline is present in the clearings-precipitate. In order to fraction free and water-soluble choline the combined filtrates are evaporated on the waterbath up to approx. 2 ml. Then choline is separated from the calciumnitrate present by an alcohol-procedure: 40 ml of alcohol are added, heated and shortly afterwards drawn off. The precipitate of calciumnitrate is again partly solved with a little water and after addition of 40 ml alcohol heated and drawn off. This procedure is again repeated. The combined alcoholic filtrates are then evaporated to dryness and the residue taken up in water and brought to a certain volume (generally 11 ml).

To 5 ml of this volume 1,5 ml potassiumtriiodide-reagens is added in order to determine free choline.

The quantity of total water soluble choline is determined by saponifying 5 ml of this solution by boiling for 1 h with 2,5 ml concentrated nitric acid, then neutralising it with calciumcarbonate and after drawing off, washing the precipitate 3 times with warm water; on evaporation of the filtrates on the waterbath the water soluble choline can be determined as enneaiodide in the remaining liquid.

Determination of the lipoid bound choline is a rather tedious procedure. For routine determinations it is sufficient to take the difference between the total and water soluble choline. It is possible to check this however:

The clearings-precipitate is treated with concentrated hydrochloric acid until the surplus of calciumcarbonate and ferrihydroxide are dissolved. On filtrating through a plyfilter the precipitate is washed free of acid and the filter together with the precipitate are then placed in a glass dish into which sulfuric acid is poured. After standing overnight at 105° water is added to the mixture under stirring; the mash obtained is neutralized with calciumcarbonate. On drawing off the precipitate is suspended in water and drawn off after boiling; this procedure is repeated once again. The combined filtrates are evaporated, boiled for 8 h with 5 ml of concentrated nitric acid, cleared with calciumcarbonate and evaporated after which the lipoid bound choline can be determined in an aliquot part.

6. DETERMINATION OF BETAINE IN THE PRESENCE OF CHOLINE

Glycine-betaine $C_5H_{11}O_2N$ $[(CH_3)_3\,N.CH_2COO^-]$ crystallizes with one mol of crystalwater; owing to a great similarity in the formulas of choline and betaine several reagentia show the same precipitation-reactions with these two compounds. In principle, however, it is possible to separate the reineckate- or the periodide compounds from the choline and betaine, as betaine has a potential carboxylgroup and choline an alcoholgroup; on account of this in a weakly alcaline or neutral solution only the choline-reineckate or -periodide compounds are precipitated, whereas in an acid environment the betaines are also precipitated. A technical difficulty on determining the betaines arises however owing to the fact that the reineckate and the periodide compounds of the betaines are more easily soluble than the corresponding compounds of choline; owing to this the separation and rinsing of the betaine-precipitates becomes more difficult.

The separation of choline from the betaines via the periodide has already been described by STANEK (124); whereas later BLOOD and CRANFIELD (20) and also REIFER (108) have worked out a determination of glycine-betaine as periodide besides choline.

According to our experience the reineckate method is not very suited for the determination of betaine owing to the relatively great solubility of the betaine-reineckate. WALKER and ERLANDSEN (145) also could not isolate the betaine-reineckate quantitatively. Quantities of 2 to 20 mg of betaine can be determined by this method with an accuracy of 4 %. Large quantities of mycelium of *Aspergillus niger* are required in order to determine betaine. That is why we have used Reifer's periodide method for our investigation. When betaine in the presence of choline was determined in the mycelium of *Aspergillus niger* it appeared that after a treatment with nitric acid according to Ducet (46) no clearly defined endpoint of titration with thiosulphate could be obtained. The reason is probably that this determination takes place in an acid environment and the traces of nitric acid still present in the clarified solution have a disturbing influence. To avoid this difficulty we neutralised with $BaCO_3$ instead of $CaCO_3$ after the nitric acid treatment; the solubility, namely of bariumnitrate is considerably less than that of calciumnitrate (resp. 8.7 at 20° and 341 at 25°). In order to remove the still present bariumnitrate we used an alcohol-treatment after the cleared filtrates had been evaporated. The determination then proceeds as follows:

1 g of dried mycelium of *Aspergillus niger* is treated with nitric acid and the extract cleared; bariumcarbonate however is used instead of calciumcarbonate to neutralize. On evaporation of the cleared liquid an alcohol treatment is used as with the determination of free choline (see page 183). After evaporation of the alcohol the residue is taken up in water, discolored with norit and filled to a given volume. In an aliquot part of this liquid choline is then determined as an enneaiodide.

To determine choline plus betaine 2 ml of the liquid is poured into a centrifuge-tube, then 1 ml of a 30% sodiumchloride solution is added, in order to reduce the solubility of betaine-periodide, and mixed. Next 0,5 ml of concentrated phosphoric acid is slowly added at the wall of the tube so that this settles as a separate layer at the bottom. This protects the subsequently formed periodide-precipitate during the rinsing. Then 1 ml of a potassiumtriiodide solution according to Reifer (108) (60 g I_2, 120 g KI and 100 g NaCl per liter water) is added and the top layer stirred with a glass rod. On standing for 3 h at approx. — 10° C, it is centrifuged as a result of which, the betaine-periodide precipitate joins the phosphoric acid layer; removal of- and rinsing of the excess reagens in the upper layer proceeds as in the choline determination. After dissolving the precipitate in alcohol the I bound by choline and betaine is titrated with sodiumthiosulfate. According to the formula of the betaine-periodide $C_5H_{11}O_2N.HI.I_5$ 5 atoms of I correspond with 1 mol betaine-HCl or 1 ml 0,0500 N thio equals 1,535 mg betaine-HCl.

On interpreting the results of the betaine-determination of Reifer (108) various difficulties arise which we will explain more fully on page 189.

Chapter III

SOME RESULTS WITH THE DETERMINATION OF CHOLINE AND BETAINE IN VARIOUS MATERIALS

1. Comparison between the choline-determination via reineckate and via enneaiodide

We deemed it advisable to determine the quantity of total choline in a number of products according to the method of Engel (50) as well as to the method of Ducet (46), because a comparison between these methods is lacking in the literature. In the first place we investigated whether the same value was acquired with the determination via reineckate in a solution of pure choline-chloride as with the determination via the enneaiodide (Table 2). Choline-chloride of Hoffmann-Laroche and choline-chloride that we isolated from the dregs of soya were used as reference substances. As choline-chloride is extremely hygroscopic the concentrations of these solutions were calculated from the N-percentages determined according to Kjeldahl. It should here be pointed out that Reifer (108) only succeeded in finding part of the theoretical quantity of nitrogen; prolongation of the destruction-period or the use of other catalysts brought no improvement. Stanek (123) and Kiesel (78) obtained the theoretically expected quantities of N in the precipitates of ennea-iodide; Termeulen and Ravenswaay (103) too found the right values. As a catalyst for the destruction we used a mixture of Se, $CuSO_4$ and Na_2SO_4; we found only a part of the total amount of nitrogen in betaine or choline. However when 200 mg glucose was added before the destruction the theoretical values were obtained.

The isolation of choline from the dregs of soya was executed as follows:

140 gr of dregs of soya was extracted with chloroform and the extract of chloroform poured out in a sixfold volume of acetone. After standing for 1 h. in the dark the lecithin was drawn off and hydrolysed by boiling 4½ h. with diluted hydrochloric acid. This hydrolysed mixture was evaporated to dryness on the waterbath and the residue extracted with 100% alcohol. After treatment with norit this filtrate was concentrated in vacuo, then diluted with water and the pH of the solution adjusted at 8. After this ammonium-reineckate (dissolved in alcohol) was added until the supernatant liquid turned dark-red. After standing in the refrigerator the choline-reineckate formed was drawn off and washed with cold alcohol. After drying the meltingpoint of the reineckate was 254° under decomposition. The choline-reineckate was decomposed by adding the calculated quantities of silver-sulphate and bariumchloride [Kapfhammer c.s. (19)]. The choline solution was brought to a certain volume.

TABLE 2

Estimation of choline as a reineckate or as an enneaiodide in a solution of pure choline-chloride

choline-chloride preparation	mg choline-chloride calculated from N-content according to Kjeldahl	mg choline-chloride precipitated as	
		reineckate	enneaiodide
isolated from the dregs of soya	1,71 1,77	1,70 1,72	1,76 1,74
Hoffmann-Laroche	2,06 2,08	2,06 2,10	2,03 2,07

Table 2 shows that the choline-estimation via the reineckate and via the enneaiodide produces equal values in a pure solution.

Thereafter we compared the hydrolysis and clearing according to Ducet (46) with the process according to Engel (50).

It appeared impossible to estimate choline as an enneaiodide after a pretreatment according to Engel as the precipitate produced by adding the potassium triiodide-reagens, did not completely dissolve in alcohol. In the liquid after the pretreatment according to Engel the with potassiumtriiodide interfering compounds are not sufficiently eliminated.

Table 3 shows the results acquired with a number of products. Both methods of estimation produced similar results.

TABLE 3

Quantities of total choline in some feeding stuffs and in the mycelium of Aspergillus niger (mg. choline-chloride per 100 g. air-dried material)

Products	Intake (g)	Pretreatment acc. to Engel (48) and estimated as reineckate	Pretreatment acc. to Ducet and Kahane (45) and estimated as	
			reineckate	enneaiodide
Lolium perenne	1	173	169	171
potatoes	1	—	120	120
steamed potatoes . . .	1	98	100	100
livermeal	0,5	857	851	821
tankage	1	270	284	273
dried carrots.	1	—	88	89
dried yeast	0,5	—	—	459
dried mycelium of *Aspergillus niger*	0,5	—	650	668

The results of a number of determinations where choline has been added to dried mycelia of *Aspergillus niger* are shown in table 4; mycelia without the addition of choline served as controls. On account of the fact that the total quantity of choline added is recovered it follows that the method of determination employed produces reliable and reproductive figures. The deviation of the quantities recovered varies from —4 to + 2 %.

TABLE 4

Recovery of the quantity of choline added to dried mycelium of Aspergillus niger. (3 ml of a solution of choline-chloride was added (1,05 mg/ml)).

Intake mycelium (mg)	mg choline-chloride in mycelium		Recovery	
	with addn. of choline (3,15 mg)	without addn. of choline	mg/ml soln.	%
500	5,34	2,30	1,01	96
400	5,05	1,88	1,06	101
400	5,76	2,68	1,03	98
400	6,32	3,17	1,05	100
400	6,10	2,89	1,07	102

2. DETERMINATION OF THE CHOLINE FRACTIONS IN HIGHER AND LOWER PLANTS

As has been mentioned before it was desirable in order to obtain some insight in the metabolism of choline in *Aspergillus niger* to determine the forms of choline which occur in the mycelium.

This separation has been made not only in the *Aspergillus niger* but also in grass, soy-flour and dried yeast; the results have been laid down in table 5:

TABLE 5

Determination of choline-fractions in grass, soy-flour, dried yeast and mycelium of Aspergillus niger. (mg choline-chloride per 100 g air-dry material)

Products	Lolium perenne		Soyflour		Dried yeast		Dried mycelium of Asp. niger	
	mg	%	mg	%	mg	%	mg	%
Total choline	175	100	231	100	459	100	572	100
Lipoid bound choline .	98	56	22,5	10	17	4	—	22
Total watersoluble choline	76,5	44	183	81	432	94	447	78
Free choline	65,5	37	127	59	15	3	18	3
Watersoluble bound choline (calculated)	—	7	—	22	—	91	—	75

On studying the values of watersoluble bound choline a marked difference is seen: it appears that the mycelia examined contain a large quantity of watersoluble bound choline (91 % of yeast and 75 % of the total amount of *Aspergillus niger*) whereas in *Lolium perenne* and soy-flour these values are 6 resp. 22 %. In so far as one is allowed to draw conclusions from these few analyses one would be inclined to suppose that in the higher plants choline is generally present in a lipoid bound form (lecithin), whereas in mycelia choline is mostly present in a watersoluble bound form. KAHANE and LEVY (74) point out that the foot of the snail *Patella vulgata* especially contains watersoluble bound choline; they have tried to isolate this form of choline in this material, but their material was lost owing to the war (75). To start a new investigation on the exact composition of watersoluble bound choline the lower plants provide a material which is easier obtained. The high percentage of watersoluble bound choline in yeast and in *Aspergillus niger* equals a low percentage of lipoid bound choline. In *Lolium perenne* we found 56 % of the total amount of choline to be lipoid bound and in soy-flour only 10 %. This low figure is probably due to the action of lecithinase C described by HANAHAN and CHAIKOFF (61) (See Chapter I,4) through which choline is separated from lecithine. We have tried to verify this in *Lolium perenne* by assessing the different fractions of choline in the fresh and artificially dried material. For comparison the fractions in an old sample dried with hot air have also been mentioned (table 6).

It appears that a similar enzyme is active in *Lolium perenne*, in fresh material the quantity of lipoid choline averages 78 %, after drying quickly at 105° 56 % and after drying more slowly in a hot current of air 27 % of the total quantity, whereas in an old sample of 1941 this only averaged 18 %.

DUCET (46) has also shown an activity of lecithinase C in potato and chestnutleaves and in the soy-plant by determining the quantity of free and lipoid bound choline in fresh and in dried material.

To investigate the action of lecithinase C in the mycelium of *Aspergillus niger* we have determined the choline fractions in fresh mycelium and in mycelium dried at 105°; the results are mentioned in table 7; the quantities of free choline are small in both cases (6 resp. 12 % of the total). The fractioning of choline during the investigation of the synthesis of choline (Chapter V) was therefore performed by us in dried mycelia because here the action of lecithinase has not been found.

TABLE 6

Influence of lecithinase on the choline fractions in Lolium perenne.
(mg choline-chloride per 100 g air-dry material)

Date Samples	30-9-1948						1941	
Pretreatment	no (fresh)		dried with hot air of ± 70° C		dried in oven at 105° C		dried with hot air of ± 70° C	
	mg	%	mg	%	mg	%	mg	%
Total choline	171	100	171	100	175	100	179	100
Lipoid choline	134	78	47	27	98	56	32½	18
Total watersoluble choline	37	22	—	73	76	44	146½	82
Free choline	—	—	—	—	66	37	116	65
Watersoluble bound choline (calculated) .	—	—	—	—	—	7	—	17

TABLE 7

The proportional relationship of the choline fractions in fresh mycelium and in mycelium dried at 105° C of Aspergillus niger of Tieghem

Days incubated	4	7	9	12	
	Fresh mycelium	Fresh mycelium	Fresh mycelium	Fresh mycelium	Dried mycelium
Total choline . . .	100	100	100	100	100
Lipoid choline (calculated) . . .	42,5	22	21	20	17
Total watersoluble choline	57,5	78	79	80	83
Free choline . . .	5	3	16	6	12
Watersoluble bound choline (calcd.) .	52,5	75	61	74	71

3. Determination of betaine in the presence of choline

In the first place the exact degree of accuracy with which small quantities of betaine can be determined was investigated. For this purpose 307,0 mg of betaine-HC1 was dissolved in 50 ml of a 30 % solution of sodium chloride; 5 ml of this solution determined by titration with lye contained 29,6 mg and calculated from the percentage of nitrogen according to Kjeldahl contained 30,7 mg betaine-HC1. Subsequently the stock solution was diluted 10 times and the quantity betaine determined according to Reifer (108) in 1,2 and 3 ml of the diluted solution (table 8). From this it is apparent that betaine-HCl can be determined up to 2 mg with a difference of 0.08 mg.

TABLE 8

Determination of betaine according to Reifer *(108)*

Intake (mg betaine-HCl)	mg betaine-HCl calculated		
	acidimetric titration	N-determination acc. to Kjeldahl	iodometric determination acc. to Reifer
30,7	29,6	30,7	—
0,61	—	—	0,61
1,23	—	—	1,15
1,84	—	—	1,81

Subsequently betaine in the presence of choline was determined in the mycelium of *Aspergillus niger*; in order to check this we also investigated whether betaine-HCl added before treatment with nitric acid and before the precipitation with potassium triiodide-reagens were recovered.

With reference to table 9 the following may be noted:

1° The quantity of betaine present in the mycelium is minute in respect to that of choline (resp. 0,77 and 4,38 mg per 400 mg dried mycelium).

2° The 1,23 mg of betaine-HCl added before precipitation was recovered quantitatively.

3° On addition of 3,69 mg betaine-HCl before the nitric acid treatment 3,64 mg has been recovered; if before the precipitation another 1,23 mg of betaine-HCl is added, the recovery amounts to 103 %.

4° The determination of betaine in the presence of choline according to Reifer's method (108) can only be used accurately if the quantity of betaine is large compared with that of choline, for betaine is found as the difference of the titration-values after precipitation in acid (betaine plus choline) and in alcaline conditions (choline). When the quantity of betaine is small in comparison with choline, the betaine is calculated from the difference between two large numbers; the result is a limited accuracy. Table 9 shows that the titration value of betaine-HCl amounts to 0,04 ml 0,05 N thio per 2 ml. A deviation of 0,02 ml in the titration value would already have given rise to a change of 50 % in the percentage of betaine of the mycelium. Moreover the interpretation of the results of the betaine estimation according to Reifer (108) gives rise to several difficulties. Besides the betaine of glycine other betaines (e.g. trigonelline) might be present. This is difficult to prove because the periodides of the betaines,—in contrast to that of choline—, do not have a typical crystalform. The presence of several betaines could be proved by way of paperchromatography. When more betaines are present the calculated value of glycine-betaine will be too high. Glycine-betaine is precipitated as a periodide with 5 titratable I-atoms, trigonelline with 3. When more betaines are present nothing can be calculated from the titration figures. We

TABLE 9

Determination of betaine and total choline in mycelium of Aspergillus niger

(400 mg mycelium pretreated acc. to Ducet (44); endvolume 25 ml; 1 ml 0,05 N thio ~ 0,875 mg choline-chloride or 1,535 mg betaine-HCl)

Intake	Choline-chloride ml thio titrated for 3 ml of the endsoln.	Choline-chloride + betaine-HCl ml thio titrated for 2 ml of the endsoln.	Betaine-HCl ml thio titrated for 2 ml of the endsoln.	mg choline-chloride per 400 mg mycelium	Mg betaine-HCl		Recovery betaine-HCl	
					per 2 ml of the endsoln.	per 400 mg mycelium	mg	%
	I	II	III = II - 2/3.I	IV=I. 0,875 $\frac{25}{3}$	V=III. 1,535	VI=III. 1,535 $\frac{25}{2}$		
A mycelium	0,60	0,44	0,04	4,38	0,06	0,77	—	—
B mycelium + 1,23 mg betaine-HCl added to 2 ml before precipitation	0,60	1,25	0,85	4,38	1,30	—	1,24[1])	101
A mycelium	0,60	0,44	0,04	4,38	0,06	0,77	—	—
B mycelium + 3,69 mg betaine-HCl before nitric acid attack	0,61	0,635	0,23	4,45	0,35	4,41	3,64[2])	99
C mycelium + 3,69 mg betaine-HCl before nitric acid attack + 1,23 mg betaine-HCl added to 2 ml before precipitation	0,61	1,465	1,06	4,45	1,63	—	1,28[3])	104

[1]) $V_B - V_A$. [2]) $VI_B - VI_A$. [3]) $V_C - V_B$.

used this method, however, for the investigation of the dissimilation of choline in *Aspergillus niger*, because under these conditions the quantities of betaine are of the same order as those of choline (see table 12 and 13); moreover under normal conditions the quantity of betaine in this mould is very small.

The results of some measurements of the quantities of choline and betaine in vegetable stuffs are given in table 10.

TABLE 10

Quantities of choline and betaine in mycelium of Aspergillus niger, in Lolium perenne and in molasses

	Choline-chloride	Betaine-HCl
Mycelium A. niger on normal nutritive solution	6,3 mmol per 100 g dry matter	0 mmol per 100 g dry matter
id. on nutritive solution [1]) 12,8 mmol added choline-chloride	6,7 mmol per 100 g dry matter	8,4 mmol per 100 g dry matter
id. on nutritive solution [2]) 11,5 mmol added betaine-HCl	6,0 mmol per 100 g dry matter	8,3 mmol per 100 g dry matter
Lolium perenne	1,2 mmol per 100 g dry matter	4,0 mmol per 100 g dry matter
Molasses.	0,3 mmol per 100 g	9,3 mmol per 100 g

[1]) In the nutritive-solution still 1,9 mmol choline-chloride and 0,9 mmol betaine-HCl present.

[2]) In the nutritive-solution still 0,0 mmol choline-chloride and 3,9 mmol betaine-HCl present.

Chapter IV

THE METABOLISM OF CHOLINE IN ASPERGILLUS NIGER: CHOLINE-OXIDASE

1. Choice of organism; method of cultivation; choline-contents and age of mycelium

In order to study the metabolism of choline in plants we chose *Aspergillus niger* of Tieghem. Preliminary investigations had shown that the percentage of choline present in this mycelium was rather high (approx. 0,6 % of the dry stuff).

The stock cultures of the mould were cultivated on an agar medium of the following composition: malt-agar (Difco) 45 g, asparagine 2 g, peptone 10 g, water 1 l. After inoculation the tubes were incubated during 3 weeks at 25° C. Afterwards a sporesuspension was made by collecting the spores of about 20 tubes in 120 ml sterile water. Cultures on a basal medium of the following composition were used: saccharose 40 g, glucose 10 g, ammonianitrate 10 g, primary potassium phosphate 5 g, magnesiumsulfate 7 H_2O 2,5 g, zincsulphate 7 H_2O 50 mg; ferrichloride trace; water 1 l. In each erlenmeyerflask of 100 ml capacity 25 ml of basal medium was brought together with 2 ml of the sporesuspension; temp. 25° C.

In very young mycelia of about 3 days old the average deviation of the dry stuff amounted to approx 6 %; in older cultures (5–7 days) the average difference is smaller (approx. 1 %). For choline-determinations mycelia of three or more flasks were dried together at 105° C.

In order to investigate the metabolism of choline during the development of *Aspergillus niger* quantities of total, watersoluble bound, lipoid and free choline were determined in a series of cultures (fig. 1).

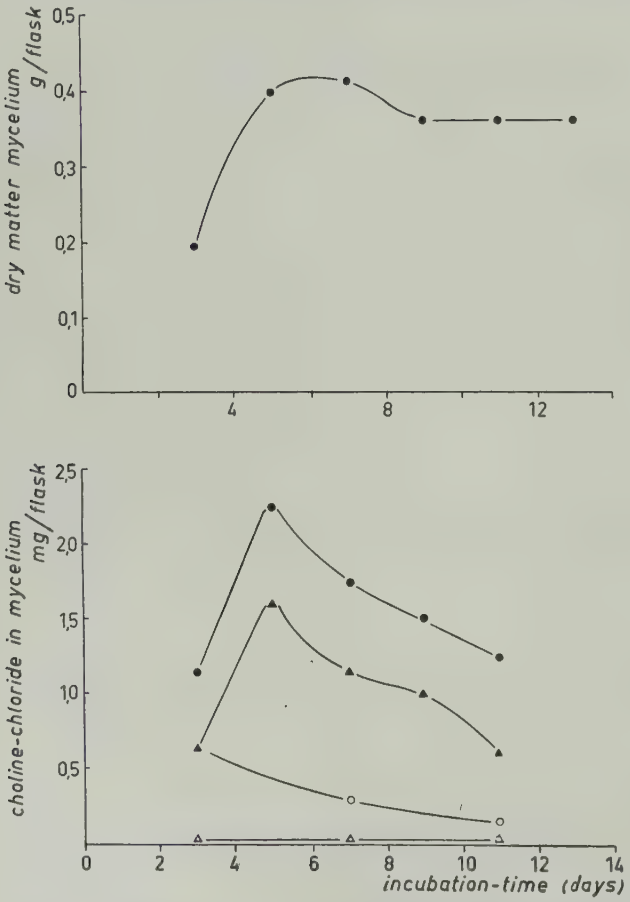

Fig. 1. Relation between the choline contents and the age of mycelia of *Aspergillus niger* (•——• total choline; ▲——▲ watersol. bound choline; ○——○ lipoid choline; △——△ free choline)

The quantities of total and watersoluble bound choline attain their maximum in the mycelia of this series after 5 days, whereafter a rapid decrease takes place. The lipoid bound choline also decreased when the mycelia grow old; the free choline is only a small part of the total choline present. Even after an incubation of 12 days the solution contained only very small quantities of choline. That is why in many cases we only determined the quantities of choline in the mycelia.

2. Investigation to the occurrence of a choline-oxidase in Asp. niger

In order to study the dissimilation of choline we took young actively growing cultures. This was done in order to prevent that part of the contents of the cell would permeate into the basal-solution. This is one of the difficulties with older cultures caused by the dying-off of part of the mycelium. The nutritive solution was always replaced by a basal solution lacking carbohydrates. The influence exerted by this impoverishment is shown in fig. 2; 24 hours after the impoverishment a synthesis of choline still takes place owing to the reserve-substances present in the mycelium. Later on the amount of total choline decreases.

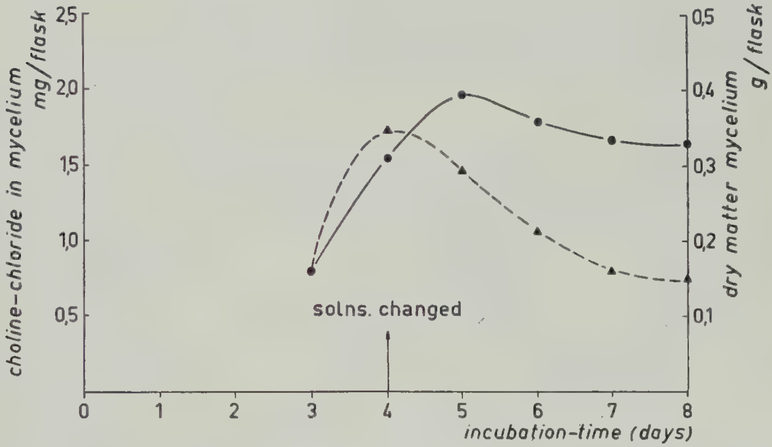

Fig. 2. Influence of changing the culture-soln. by a basal-soln. lacking carbohydrates on the choline contents of mycelia of *Aspergillus niger* (•——• total choline; ▲----▲ dry matter)

As during the impoverishment the quantity of dry material of the mycelium decreases much more than the amount of choline it is probable that this group of compounds is not easily taken up in the metabolism.

We investigated the dissimilation of choline by adding free choline to the basal solution without carbohydrates. After an incubation period of 97 hrs. the culture liquid was replaced by a basalsolution without carbohydrates; moreover choline was added to some of the cultures. Fig. 3 shows that the greater part of the choline added (1,9 mg) disappeared from the solution after 25 hours, whereas the total percentage of choline of the mycelium is only slightly more (0,3 mg) than that of the control cultures. The added choline is apparently not aerobic converted into watersoluble- or lipoid bound choline.

It has been seen from literature mentioned in Chapter I that the most likely reactions during the dissimilation of choline are an oxidation to betaine or a degradation under separation of trimethylamine. We have first investigated whether the added quantity of choline was converted into betaine.

Some preliminary experiments showed that this was indeed the case.

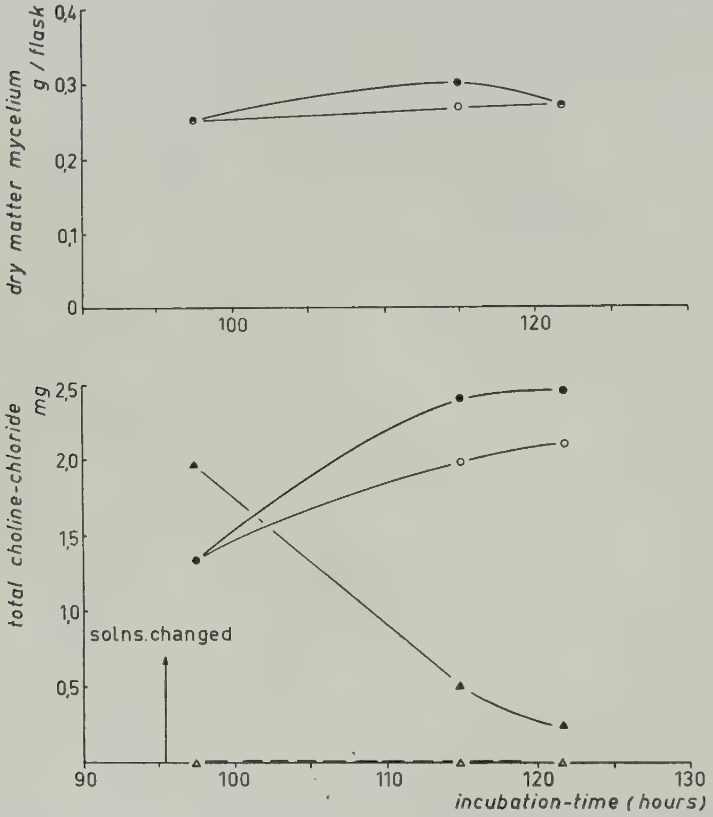

Fig. 3. Choline contents of mycelia and nutritive-solns. of cultures of *Asp. niger* after impoverishment in carbohydrates at 97 h. (•——• with choline; ○——○ without choline; ▲——▲ choline in nutr. - soln. after addn.; △----△ id. without addn.)

Table 11 shows the results of a series of experiments performed with the aim to investigate the conversion of choline in betaine through *Aspergillus niger*. The liquid under the mycelium in a number of flasks was replaced after an incubation of 120 hrs. by a solution without carbohydrates; moreover in the series II, III and IV resp. 0,055 mmol choline-chloride, 0,050 mmol betaine-HCl and 0,055 mmol choline-chloride plus 0,050 mmol betaine-HCl was added. The addition of betaine was necessary in order to investigate the possibility that the betaine formed from choline was converted afterwards into other compounds. Besides choline betaine was determined too in the mycelium and in the culture medium.

With regard to table 11 the following may be noted:

1. The quantity of choline plus betaine has increased in 24 hours

of incubation after the solution has been renewed from 0,021 to 0,026 mmol per flask (series I). It is not absolutely certain that this is also the case in series II, III and IV where choline and (or) betaine was added. For simplicity's sake we based our calculation of the total number mmol in the last three series on the figures of series I; we suppose therefore that the normal weak choline synthesis is not influenced by additions. If this supposition should not be satisfactorily complied with, its influence is not of great importance because the

TABLE 11

Conversion of choline into betaine by A. niger on a basalsolution lacking carbohydrates. (After 120 hrs. of incubation a new basalsolution without carbohydrates was brought under the mycelium; moreover in series II, III and IV resp. choline-chloride, betaine-HCl and choline-chloride plus betaine-HCl was added, thereafter incubated for 24 hrs.; quantities in mmol per flask; for each determination 4 flasks were taken)

	dry matter (g)	choline-chloride (mmol)	betaine-HCl (mmol)	total mmol per flask	total mmol calcd. with series I
Quantities when the solutions are changed (120 h).					
mycelium	0,423	0,018	0,000	0,021	
nutritive solution . .	—	0,000	0,003		
Series I: quantities 24 h after changing the nutritive solutions.					
mycelium	0,397	0,024	0,000	0,026	—
nutritive solution . .	—	0,000	0,002		
Series II: as series I plus 0,055 mmol choline-chloride per flask					
mycelium	0,431	0,029	0,036	0,077	0,081 (0,026 + 0,055)
nutritive solution . .	—	0,008	0,004		
Series III: as series I plus 0,050 mmol betaine-HCl per flask					
mycelium	0,436	0,026	0,036	0,079	0,076 (0,026 + 0,050)
nutritive solution . .	—	0,000	0,017		
Series IV: as series I plus 0,055 mmol choline-chloride plus 0,050 mmol betaine-HCl per flask					
mycelium	0,415	0,034	0,036	0,129	0,131 (0,026 + 0,055 + 0,050)
nutritive solution . .	—	0,030	0,029		

largest deviation which might result can at most be 10 % of the added quantities of choline or betaine. When we compare the results in table 11 of the last two columns it follows that on acceptance of the above suppositions within the experimental deviations the quantities of choline and betaine balance. This is the reason why we stuck to this supposition as a basis for future investigation.

2. On addition of 0,055 mmol choline-chloride the total quantity of choline plus betaine per flask amounts to 0,077 mmol; on the basis of series I the calculated amount is 0,081 mmol. We may therefore conclude that choline is oxidised by *Aspergillus niger* to betaine exclusively and that no demonstrable degradation to trimethylamine takes place. We can therefore limit our studies on the dissimilation to the determination of choline and betaine. After addition of 0,055 mmol of choline-chloride 0,040 mmol of betaine-HCl is found in the mycelium and nutritive solution, whereas this quantity amounts to 0,002 for the control series. Within 24 hours *Aspergillus niger* can convert approx. 70 % of the choline into betaine.

3. The fact that the experiment described in this table produces a square balance for the dissimilation of choline proves that given these chosen experimental conditions betaine is not further converted by *Aspergillus niger*. For in series III 0,050 mmol of betaine was added, whereas 0,053 – 0,002 = 0,051 mmol (per flask) was recovered.

4. In series IV, where choline and betaine are added, the oxidation of choline is smaller than after addition of choline alone. The choline taken up amounts to 0,055 – 0,030 = 0,025 mmol, whereas this quantity amounts to 0,055 – 0,008 = 0,047 mmol in series II. This difference might be explained by supposing that the wall of the cell becomes less permeable to choline when betaine has been added simultaneously; or that owing to the presence of betaine the surface of the choline-oxidising enzymesystem is already so covered by materials which have a quaternaire ammonia-group that the choline has less chance of oxidising. MANN c.s. (97) thus account for the reduced oxidation of choline by an enzyme preparation from ratliver in the presence of materials with $-NH_3$ groups as NH_4^+, $NH(CH_3)_3^+$ and betaine.

Then we investigated if cultures, who were not impoverished in carbohydrates, oxidized the added choline in the same way (table 12)

Owing to the presence of carbohydrates in the new basalsolution the mycelia have grown rapidly after the renewal, whereas in 48 h. after renewal the quantity of choline was doubled. If one wishes to suppose that normal synthesis is not influenced by the addition of choline or betaine, the added choline is practically recovered as choline + betaine, in other words, no other reaction would take place except the conversion of choline into betaine. The increase of betaine in this series compared to the control determination amounts to 0,046 – 0,003 = 0,043 mmol, which is a conversion of approx. 93 % of the quantity of choline taken up. In series III where 0,040 mmol betaine-HCl per flask has been added this is recovered practically unchanged (0,044 mmol). It seems that in the presence of carbohydrates betaine does not react under the experimental conditions.

We may conclude from table 12 that notwithstanding the presence of carbohydrates oxidation proceeds more rapidly than the incorporation to watersoluble bound choline. Possibly the glycero phosphoricacid is not present in sufficient quantities to bind the choline at the right time. A relatively greater binding could be ensured if choline was added in equal quantities as with which glycerine-phosphoricacid is produced during the metabolism.

TABLE 12

Oxidation of choline by Asp. niger to betaine on a basal-solution containing carbohydrates. (after 144 hrs of incubation a new basalsolution containing carbohydrates was brought under the mycelium (series I) which in series II and III also contained choline-chloride and betaine-HCl. Thereafter incubated for 48 h; quantities in mmol per flask; for each determination 3 flasks were taken)

	dry matter (g)	choline-chloride (mmol)	betaine-HCl (mmol)	total mmol per flask	total mmol calcd. with series I
Quantities when the solutions are changed (144 h)					
mycelium	0,491	0,013	0,003		
nutritive solution . .	—	—	—		
				0,016	—
Series I: quantities 48 h after changing the nutr. solns.					
mycelium	0,905	0,027	0,003		
nutritive solution . .	—	—	—		
				0,030	—
Series II: as series I plus 0,066 mmol choline-chloride per flask					
mycelium	0,898	0,026	0,020		
nutritive solution . .	—	0,020	0,026		
				0,092	0,096 (0,030 + 0,066)
Series III: as series I plus 0,040 mmol betaine-HCl per flask					
mycelium	0,911	0,027	0,011		
nutritive solution . .	—	—	—		
				0,074	0,070 (0,030 + 0,040)

As has already been mentioned in chapter I MANN and QUASTEL (95) have observed the oxidation of choline under anaerobic conditions. They found that the choline-oxidising enzymesystem in rat-liver consisted of at least 2 parts: a cytochrome—and indolphenoloxidase and a choline-dehydrogenase, which is not inhibited by small quantities of cyanide. Nevertheless they succeeded in obtaining an oxidation of the choline under anaerobic conditions through this dehydrogenase when a suitable hydrogen acceptor i.c. sodium-ferricyanide was added. Analogous with these experiments we have investigated the oxidation of choline by *A. niger* under anaerobic conditions. For this experiment the flasks were placed in a vacuum-exsiccator after the nutritive solution had been renewed. After evacuation N_2 was induced after having removed all traces of oxygen by rinsing in a solution of sodium-hydrosulphate. The evacuation and inducement of nitrogen was once more repeated and after incubating for 24 h the quantity of choline was determined. Table 13 and 14 show the results of these experiments.

The results of the experiments described in table 13 prove that

TABLE 13

Oxidation of choline by A. niger under aerobic and anaerobic conditions. (nutritive solution lacking carbohydrates)

	dry matter (g)	choline-chloride (mmol)	total mmol choline-chloride per flask	increase compared with the quantities when the solns. are renewed
Quantities when the solutions are changed. (incubated for 97 h)				
mycelium	0,271	0,009	0,009	—
nutritive solution	—	—		
Aerobic.				
1. Quantities 25 h after changing the nutritive solutions. (0,014 mmol choline-chloride added per flask)				
mycelium	0,277	0,018	0,020	0,011
nutritive solution	—	0,002		
2. as 1 but without choline-chloride-addn.				
mycelium	0,262	0,015	0,015	0,006
nutritive solution	—	0,000		
			mmol choline disappeared 0,009	
Anaerobic.				
1. Quantities 25 h after changing the nutritive solutions. (0,014 mmol choline-chloride added per flask)				
mycelium	0,272	0,011	0,022	0,013
nutritive solution	—	0,011		
2. As 1 but without choline-chloride-addn.				
mycelium	0,258	0,010	0,011	0,002
nutritive solution	—	0,001		
			mmol choline disappeared 0,003	

TABLE 14

Oxidation of choline by A. niger under aerobic and anaerobic conditions (nutritive solutions lacking carbohydrates)

	dry matter (g)	choline-chloride (mmol)	total mmol choline-chloride per flask	increase compared with the quantities when the solns. are renewed
Quantities when the solutions are changed (149 h)				
mycelium	0,544	0,023		
nutritive solution . .	—	—	0,023	—
Aerobic.				
1. Quantities 24 h after changing the nutritive solutions (0,046 mmol choline-chloride added per flask)				
mycelium	0,482	0,027		
nutritive solution . .	—	0,002	0,029	0,006
2. As 1 but without choline-chloride-addn.				
mycelium	0,467	0,024		
nutritive solution . .	—	—	0,024	0,001
			mmol choline disappeared 0,041	
Anaerobic				
1. Quantities 24 h after changing the nutritive solutions (0,046 mmol choline-chloride added per flask)				
mycelium	0,420	0,016		
nutritive solution . .	—	0,050	0,066	0,043
2. As 1 but without choline-chloride-addn.				
mycelium	0,410	0,012		
nutritive solution . .	—	0,011	0,023	0,000
			mmol choline disappeared 0,003	

after incubation during 25 h from the added 0,014 mmol of choline-chloride under aerobic conditions 0,020 – 0,015 = 0,005 mmol are still present; under anaerobic conditions rests 0,022 – 0,011 = 0,011 mmol. This means that 0,009 resp. 0,003 mmol of choline-chloride have disappeared.

Still clearer figures were obtained with the experiment mentioned in table 14, which was conducted in a similar way as that in table 13; the quantity of choline-chloride added however is much larger viz. 0,046 mmol. After incubating for 24 h. under aerobic conditions 0,006 mmol is left as choline-chloride, whereas under anaerobic conditions this amounts 0,043 mmol; resp. 0,041 and 0,003 mmol choline-chloride disappeared.

A strong oxidation of choline therefore occurred under aerobic conditions. The decline observed under anaerobic conditions (0,003

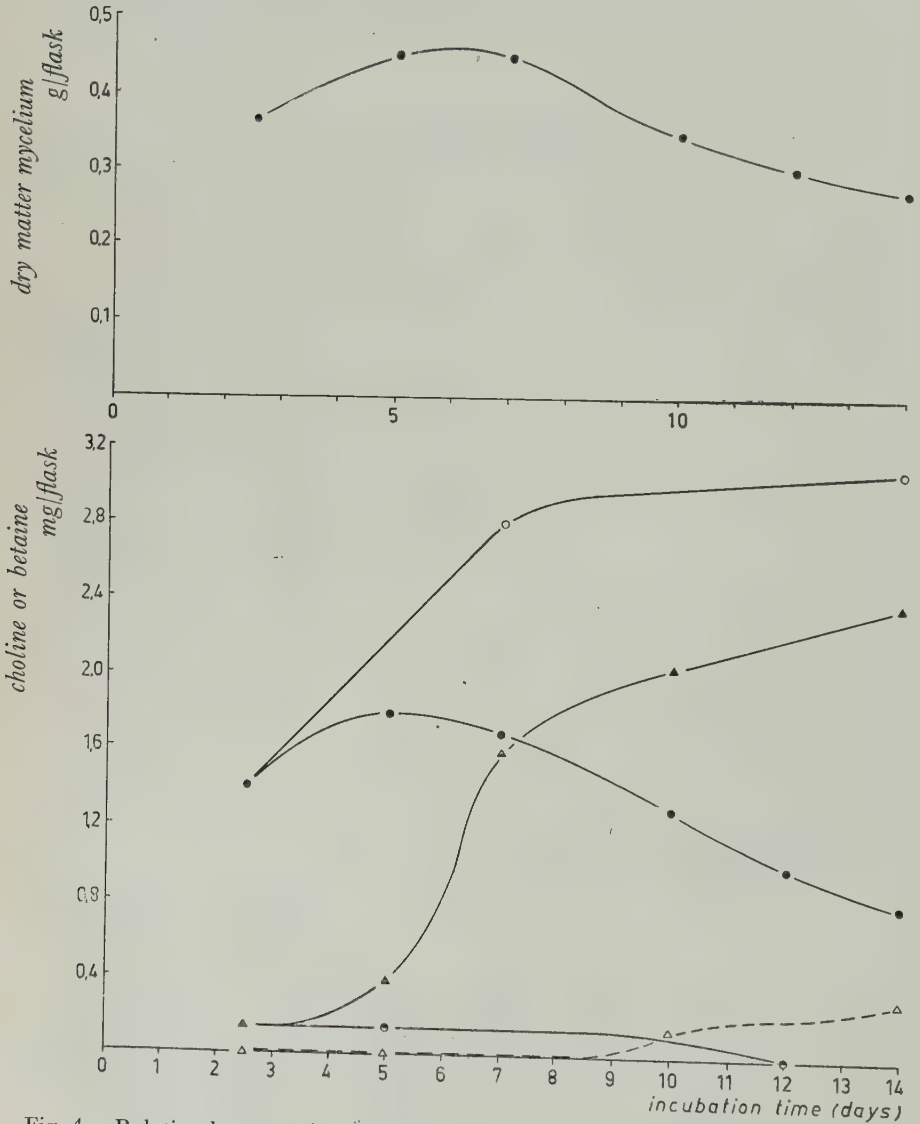

Fig. 4. Relation between the choline and betaine contents and the age of cultures of *A. niger* (•——• choline per flask; ◓——◓ id. in soln. per flask; ▲——▲ betaine per flask; △----△ id. in soln. per flask; ○——○ choline and betaine per flask)

mmol) can not be considered as significant because this figure is of the same magnitude as the experimental deviation. During the experiments shown in table 14, the quantity of choline in the mycelia under anaerobic conditions was reduced half of the original amount, where as in the experiments of table 13 the quantity even slightly increased under anaerobic conditions. Both cases showed no clear dehydrogenation of choline under anaerobic conditions.

Finally it should be noted that we tried to obtain an anaerobic oxidation of the choline by adding potassium-ferricyanide as a hydrogen-acceptor. This compound had a poisonous effect in the concentrations used (0,55 and 0,09 %) owing to which the largest part of the choline (± 75 %) disappeared from the mycelium into the nutritive solution. No oxidation of choline could be established.

On investigating the metabolism of choline in relation to the development of *A. niger* we found that the quantity of total choline attained its maximum after 5 days, whereafter a rather rapid decrease occurs (fig. 1). The preceding pages have shown that choline can be practically quantitatively oxidised to betaine. Next we investigated whether the decrease of the amount of choline on the fifth day during normal growth was compensated by an increase of the quantity of betaine. The results of such an experiment* are mentioned in fig. 4 where the quantity of choline and betaine present in the mycelium and in the nutritive solution were determined after $2^1/_2$, 5, 7, 10, 12 and 14 days. The quantity of choline and betaine present in the nutrition solution was again very small. The experiment shows that the decrease of the quantity of choline correlates with an increase of the quantity of betaine. The quantity of choline + betaine after 7 days is larger than after 5 days, whereas the amount of choline has already been decreased: apparently between the 5th and 7th day choline is still synthesized under simultaneously oxidation to betaine.

The total quantity of choline and betaine remains more or less constant after the 7th day. At this stage the metabolism of choline results in a liberation of choline from the watersoluble bound choline by lecithinase and in an oxidation of the choline to betaine.

Chapter V

THE METABOLISM OF CHOLINE IN ASPERGILLUS NIGER: SYNTHESIS

1. Synthesis from ethanolamine and methionine

The investigation of the synthesis of choline in plants has been limited to 2 groups of investigations: the methylation of ethanolamine by methionine in a mash of wheat seedlings, whereby Barrenscheen and collaborators (8) meant to establish a synthesis of choline. Steensholt however (126) could not confirm this. Horowitz (63,

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* For the execution of these experiments we are especially grateful to Mr. W. Graafland, biol. drs.

64) investigated the in vivo synthesis of choline in *Neurospora crassa* by comparing the growth of choline-less mutants. Recently KIRKWOOD (79, 99) showed that when $HC^{14}OONa$ or l-methionine labelled with C^{14} in the methylgroup was fed to sprouting barley, the methylgroups of choline contained C^{14}.

During our investigation on the synthesis of choline we replaced the old nutritive solution for a new one to which ethanolamine or methionine had been added. We started with the investigation of the activities of these two compounds as precursors in the synthesis of choline. Table 15 clearly shows that *Aspergillus niger* does not synthesize choline from these 2 compounds on a solution without carbohydrates.

When however the solution under the mycelium is replaced by a nutritive-solution containing carbohydrates, addition of methionine plus ethanolamine produces a distinct synthesis (Table 16).

On addition of methionine plus ethanolamine approx. 0,039 mmol of choline has been recovered in the mycelium; without addition 0,025 mmol, so that the synthesis is 0,014 mmol choline per flask, i.e. approx. 14 % of the theoretically possible quantity. Besides the quantity of choline in the nutritive solution has been determined in a number of these series; this was very slight however, so that these values are not mentioned in a number of experiments.

After we had succeeded in obtaining a synthesis from methionine plus ethanolamine it was of importance to investigate whether it was sufficient for this synthesis to add one of these compounds; this might indicate a surplus presence of the other compounds in the cell.

Moreover it could be decided whether the influence of methionine and ethanolamine is additive or whether the quantity of choline produced from both compounds is larger than the sum of the synthesis acquired on adding each of these compounds separately (table 17)

TABLE 17

Choline-synthesis by A. niger on a nutritive-solution containing carbohydrates after adding methionine, ethanolamine, methionine plus ethanolamine

Added compound	Synthesis (mmol choline-chloride per flask)		
	average	maximum and minimum value	number of series
0,248 mmol methionine	+ 0,001	— 0,003; + 0,003	5
0,098 mmol ethanolamine	— 0,001	— 0,005; + 0,003	4
0,248 mmol methionine + 0,098 mmol ethanolamine	+ 0,013	+ 0,010; + 0,018	7

The added quantities of methionine and ethanolamine are the same as those in table 16. Owing to the rather large deviation we were obliged to repeat these experiments several times. The final column shows the number of series out of which the averaged figure has been calculated. It appears that on addition of methionine or of ethanolamine

TABLE 15

Synthesis of choline from methionine and ethanolamine by Aspergillus niger on a nutritive-solution lacking carbohydrates.
(every figure originates from at least 3 flasks).

Series	h incubated before changing nutritive-soln.	h incubated after changing nutritive-soln.	added per flask		dry matter of mycelium (g per flask)		choline-chloride (mmol per flask)		Synthesis
			ethanol-amine mmol	methionine (mmol)	flasks with addn.	flasks with-out addn.	flasks with addn.	flasks with-out addn.	
I	132	30	0,014	0,042	0,239	0,264	0,012	0,012	0,000
	132	72	0,014	0,042	0,167	0,197	0,012	0,012	0,000
II	140	24	0,076	0,225	0,398	0,407	0,024	0,026	—0,002
III	143	48	0,098	0,242	0,408	0,425	0,015	0,014	+0,001
IV	147	37	0,098	0,242	0,440	0,465	0,014	0,016	—0,002

TABLE 16

Synthesis of choline from methionine and ethanolamine by Aspergillus niger on a nutritive-solution containing carbohydrates.
(every figure originates from at least 3 flasks)

Series	h incubated before changing nutritive-soln.	h incubated after changing nutritive-soln.	added per flask		dry matter of mycelium (g per flask)		choline-chloride (mmol per flask)		Synthesis
			ethanol-amine mmol	methionine mmol	flasks with addn.	flasks with-out addn.	flasks with addn.	flasks with-out addn.	
I	148	39	0,098	0,248	0,727	0,742	0,042	0,027	0,015
II	143	38	0,098	0,248	0,913	0,825	0,041	0,023	0,018
III	147	37	0,098	0,248	0,904	0,806	0,037	0,024	0,013
IV	144	48	0,098	0,248	1,018	0,905	0,037	0,027	0,010
						Average	0,039	0,025	0,014

no choline synthesis occurs. Both methionine and ethanolamine are necessary to produce a synthesis of choline.

In order to acquire data on what form choline synthesized from methionine and ethanolamine is present, we have determined several choline fractions in mycelia acquired from a synthesis-experiment. Choline appeared to be present in a practically equal percentage in the watersoluble form as in the mycelia of control-cultures (table 18). The additional synthesized choline is present in at least 70–80 % as watersoluble bound choline in the mycelium.

If we bear in mind the possibility of a slight deviation, we see that in the newly formed choline practically the same percentages of free choline and watersoluble bound choline are present as originally in the mycelium.

TABLE 18

Division of choline in mycelia of A. niger during synthesis-experiments.
(each figure originates from 4 flasks)

	Total choline	watersoluble bound choline		During the experiment newly formed choline		
				Total choline	watersoluble bound choline	% total choline
	mmol	mmol	% total	mmol	mmol	
without addn.	0,027	0,025	93	—	—	—
after addn. (0,248 mmol meth. + 0,098 mmol ethanolamine)	0,042	0,036	86	0,015	0,011	73
after addn. (0,248 mmol meth. + 0,098 mmol glycine)	0,042	0,037	88	0,015	0,012	80

The fact that on a nutritive-solution lacking carbohydrates no increase of the quantity of choline can be seen after adding methionine and ethanolamine might be explained by supposing, that choline would have been formed but that owing to a lack of glycerophosphoric acid it is not transformed into the watersoluble bound form but directly oxidised to betaine. We have tried to verify this in two ways: 1) by determining betaine in the presence of choline 2) by adding glycerophosphoric acid.

ad 1) Table 19 proves that the total quantity of choline plus betaine without addition of methionine and ethanolamine in cultures on a solution without carbohydrates is practically equal to the quantity of choline and betaine after addition of these compounds. It should be noted that the betaine-determination can only be used here with restriction; betaine is found as a difference (see chapter III, 3) and as the quantity of choline in this experiment is large with regard to that of betaine, the values for betaine are less accurate than was the case in the investigation on choline-oxidase. The difference of 0,003 mmol therefore lies within the limit of experimental errors. The fact that no increase of the quantity of choline was noted on a nutritive-solution with methionine and ethanolamine but without carbohydrates is not due therefore to a possible further oxidation to betaine but to the fact that choline was not formed at all.

TABLE 19

Synthesis of choline by A. niger on a nutritive-solution lacking carbohydrates

	choline-chloride mmol per flask	betaine-HCl mmol per flask	total mmol per flask	increase compared with the control-flasks
Incubated for 140 h				
mycelium	0,021	0,007		
nutritive-solution . . .	—	—	0,028	—
Quantities 24 h after the nutritive-solutions are changed (0,025 mmol ethanolamine + 0.076 mmol methionine)				
mycelium	0,026	0,005		
nutritive-solution . . .	0,001	0,001	0,033	— 0,003
Quantities 24 h after the nutritive-solutions are changed without addn.				
mycelium	0,024	0,011		
nutritive-solution . . .	0,001	0,000	0,036	—

ad 2) A nutritive-solution in which the carbohydrates were substituted by glycerophosphoric acid was used. Choline synthesized from methionine and ethanolamine could possibly be converted to the watersoluble form of choline (table 20); the result is clearly negative. With carbohydrates the synthesis amounts to 0,013 mmol per flask, with glycerophosphoric acid the total quantity of choline is 0,001 mmol and without carbohydrates 0,002 mmol per flask. During the experiments we added the glycero-

TABLE 20

Synthesis of choline by A. niger on a nutritive-solution without carbohydrates, id. on a nutritive-solution with glycerophosphoric acid and on a nutritive-solution with carbohydrates. (incubated for 147 h., after renewal of nutritive-solution incubated for another 37 h., mmol choline-chloride in mycelium per flask)

Nutritive-solution after changing	Quantities when the nutritive-solutions are changed (147 h)	37 h after changing nutritive-solution		
		0,248 mmol methionine + 0,098 mmol ethanolamine	without addition	difference
—	0,014	—	—	—
without carbohydrates. .	—	0,016	0,014	+ 0,002
glycerophosphoric acid [1])	—	0,016	0,015	+ 0,001
with carbohydrates [2]) . .	—	0,037	0,024	+ 0,013

[1]) 55 g sodium-salt of glycerophosphoric acid per l.

[2]) 40 g sucrose + 10 g glucose per l.

phosphoric acid in two parts, because the possibility that this compound is dephosphorylated by the phosphatases in *A. niger* could not be ruled out: one part during the renewal of the nutritive solution and the remainder 24 h. later.

2. SYNTHESIS FROM COMPOUNDS OTHER THAN METHIONINE AND ETHANOLAMINE

Experiments on the synthesis of choline in animals had shown that other compounds than methionine and ethanolamine may function as a methyldonor and a methylacceptor. Thus ethanolamine might be replaced by monomethylethanolamine, dimethylethanolamine, glycine and serine.

This last compound according to ARNSTEIN (42) might function not only as a methylacceptor but also as a methyldonor; methionine can be replaced by betaine, dimethylthetine and dimethylpropiothetine.

Several of these compounds have been examined for their activity with regard to the synthesis of choline in *A. niger*. Table 21 shows the results of synthesis-experiments on a nutritive-solution with carbohydrates to which serine, ethanolamine + serine, serine + methionine, glycine + methionine have been added.

TABLE 21

Synthesis of choline by Aspergillus niger on a nutritive solution with carbohydrates after addition of serine, ethanolamine + serine, serine + methionine, glycine + methionine

Added compound	Synthesis (mmol choline-chloride per flask)		
	average	maximum and minimum value	number of series
0,098 mmol serine	— 0,001	— 0,005; + 0,003	3
0,098 mmol ethanolamine + 0,248 mmol serine	+ 0,005	+ 0,003; + 0,006	3
0,098 mmol serine + 0,248 mmol methionine	+ 0,002	+ 0,000; + 0,006	4
0,098 mmol glycine + 0,248 mmol methionine	+ 0,010	+ 0,008; + 0,015	2

The synthesis due to serine amounted —0,001 mmol; after addition of serine and methionine +0,002 mmol (after decarboxylation of serine ethanolamine arises), while after addition of ethanolamine and serine (serine as methyldonor) it was 0,005 mmol. These increases are too small to conclude to a synthesis.

Addition of methionine and glycine resulted in a synthesis of the same magnitude as that of methionine and ethanolamine (resp. 0,010 and 0,014 mmol). This can be explained by the supposition that *Aspergillus niger* is able to reduce glycine to ethanolamine. Another explanation is that a possible oxidation of ethanolamine to glycine is inhibited by addition of this to the basalsolution.

3. HYPOTHESES REGARDING THE RELATION BETWEEN THE SYNTHESIS OF CHOLINE AND THE CARBOHYDRATE METABOLISM

In chapter I we discussed the reaction-mechanisms for the transfer of the methylgroup of methionine to ethanolamine.

A. According to BARRENSCHEEN c.s. (5) this transfer would only take place after the oxidation of methionine to methionine-sulfoxide.

In order to investigate the course of the methylation-reaction in *Aspergillus niger* we have added methionine-sulfoxide, ethanolamine plus methionine-sulfoxide[1] and ethanolamine plus methionine to a number of cultures on a nutritive solution with carbohydrates. Table 22 shows that the quantity of choline due to the addition of methionine-sulfoxide and of ethanolamine plus methionine-sulfoxide is too small (resp. 0,004 and 0,003 mmol) to decide on a synthesis.

TABLE 22

Synthesis of choline by Aspergillus niger on a nutritive-solution with carbohydrates after addition of methionine-sulfoxide and methionine-sulfoxide plus ethanolamine

Added compound	Synthesis (mmol choline-chloride per flask)		
	average	maximum and minimum value	number of series
0,248 mmol methionine-sulfoxide . . .	+ 0,004	+ 0,003; + 0,006	3
0,098 mmol ethanolamine + 0,248 mmol methionine-sulfoxide . .	+ 0,003	+ 0,002; + 0,005	2
0,098 mmol ethanolamine + 0,248 mmol methionine	+ 0,013	+ 0,010; + 0,018	7

Following the experiments of BARRENSCHEEN on muscle extract from the rat (7) we have tried to obtain an anaerobic synthesis of choline from methionine-sulfoxide plus ethanolamine. In the event of a positive effect this would prove the reaction-mechanism formulated by BARRENSCHEEN. Besides this it was also necessary to demonstrate that under these conditions no choline synthesis resulted from the addition of methionine plus ethanolamine. Under anaerobic conditions however the fungus died in the course of this series of experiments.

The fact that choline cannot be synthesized from methionine-sulfoxide plus ethanolamine under aerobic conditions (table 22) may be regarded as an argument against the course of the choline-synthesis according to BARRENSCHEEN.

B. CANTONI (29) has shown that the transfer of the methylgroup of methionine occurs after this compound is converted by ATP in S-adenosyl-methionine. We have tried to prove the influence exerted by ATP during the methylation of ethanolamine as follows: According

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1) Methionine-sulfoxide was prepared by oxidation of methionine with H_2O_2 according to TONNIES and KOLB (132); the product acquired had a melting-point of 225° C (lit. 225-230° C).

to CHUGTAI and WALKER (35) the carbohydrate consumption of *Aspergillus niger* is increased after addition of sodium-arsenite. Analogous to experiments with yeast they assume that in the presence of sodium-arsenite the balance of the enzymatic activities would be deflected towards citrate formation. The results of their experiments prove that 0,004 M sodium-arsenite decreases respiration whereas the quantity of citric acid is considerably larger than in cultures without arsenite. Owing to this change in the metabolism a smaller quantity of ATP may be expected than in cultures without arsenite. An indirect indication to the reaction course according to CANTONI, might be acquired if it could be proved that the choline-synthesis of methionine and ethanolamine in the presence of sodium-arsenite was smaller than in cultures without arsenite. Table 23 shows the results of two experiments regarding the influence of sodium-arsenite on the synthesis of choline.

In series I the decrease caused by sodium-arsenite of the synthesis from ethanolamine and methionine amounted to 0,008–0,003 = 0,005 mmol i.c. 62 % The quantity of choline synthesized via the control cultures is considerable larger than when arsenite is present (resp. 0,033 and 0,020 mmol). Even without addition of ethanolamine and methionine sodium-arsenite apparently retards the synthesis of choline; in this case the fungus has, as N-source for the choline to be synthesized, ammonia-nitrate in the nutritive-solution at its disposal. In order to keep the choline-synthesis from the N-compounds of the nutritive-solution as small as possible, the ammonia-nitrate has been omitted from the new solution, brought under the mycelium after 102 and 118 h of incubation. It appeared then that the quantities of choline of the control cultures deprived of N are much less (resp. 0,021 and 0,019 mmol); the inhibition of the choline-synthesis from ethanolamine and methionine by sodium-arsenite amounts to 0,012–0,007 = 0,005 mmol or 42 %. The complication to series I viz. that control cultures with choline synthesized from the nutritive-solution have been used to calculate the inhibition of the choline-synthesis from methionine and ethanolamine, does not apply to series II.

The fact that arsenite inhibits the synthesis of choline, indirectly supports the view that in *Aspergillus niger* the transfer of the methylgroup of methionine is accomplished via S-adenosyl-methionine.

Another way to demonstrate the relation existing between the transmethylation and ATP can be deduced from BONNER's observation (21), that 3,4-dinitrophenol (DNP) functions as inhibitor of ATP in *Avena* coleoptiles. BACCARI and GUERRITORE (3) thus proved that the methylation of guanido acetic acid in slices or homogenates of rat-livers is inhibited by 0,004 M DNP. By administering DNP to the nutritive-solution of cultures of *Aspergillus niger* we now tried to reduce the action of ATP; after the addition of ethanolamine and methionine this would result in finding no or only a minute choline-syntheses. The results of these experiments however varied so much that we were unable to draw any conclusions.

TABLE 23

The influence of 0,004 M sodium-arsenite on the synthesis of choline from methionine and ethanolamine on a nutritive-solution containing carbohydrates (mmol choline-chloride in mycelium per flask; each figure originates from 3 or 4 flasks).

Series		0,098 mmol ethanolamine + 0,248 mmol methionine		Without methionine + ethanolamine		Syn-thesis mmol	Inhibition of the synthesis	
		dry matter (g)	mmol choline-chloride	dry matter (g)	mmol choline-chloride		mmol	%
I	Nutr.-soln. changed after 103 h; incubated for 47 h,	—	—	0,709	—	—	—	—
	again new nutr.-soln. and still incubated for 92 h							
	without sodium-arsenite	0,873	0,041	0,896	0,033	0,008		
	with sodium-arsenite	0,828	0,023	0,819	0,020	0,003		
							0,005	62
II	Nutr.-soln. changed after incubating for 102 h,	—	—	0,591	—	—	—	—
	impoverished in N for 16 h; again new nutr.-soln.							
	without N and still incubated for 48 h							
	without sodium-arsenite	0,849	0,033	0,848	0,021	0,012		
	with sodium-arsenite	0,818	0,026	0,727	0,019	0,007		
							0,005	42

DISCUSSION

The experiments in chapter IV and V have shown that *Aspergillus niger* oxidises added free choline to betaine; when carbohydrates are present the addition of free choline does not result in an incorporation into a water-soluble bound form. The dissimilation of choline is apparently not influenced by the metabolism of carbohydrates.

This does happen, however, at the synthesis of choline. The formation of choline from methionine and ethanolamine occurs only in the presence of carbohydrates whereafter choline is converted into the water-soluble form. Without carbohydrates no increase of the quantity of choline or secondary formed betaine was observed. The addition of glycerophosphoric acid which would promote the binding of eventually formed choline, was of no effect.

The choline synthesis can schematically be divided in 2 phases: the methylation of ethanolamine and the incorporation of choline to watersoluble bound choline. What connection exists between the metabolism of carbohydrates and each of these phases?

For the transfer of the methylgroup to ethanolamine two reaction-mechanisms have been described [Barrenscheen (5) and Cantoni (29)]; regarding the formation of the watersoluble bound choline no data are known.

It might be possible to find a connection existing between the metabolism of carbohydrates and the choline synthesis if one regards the oxidation of methionine to methionine-sulfoxide [Barrenscheen (5)] linked with the reduction of phosphoglyceraldehyde to glycerophosphoric acid. In view of the negative results obtained with methionine-sulfoxide in table 22 the course of the reaction formulated by Barrenscheen in *Aspergillus niger* is not very probable.

Although we are only able to prove indirectly the connection between ATP and the transfer of the methylgroup of methionine to ethanolamine as described by Cantoni (29), this view is in preferable to that of Barrenscheen. Baccari and Guerritore (3), with regard to the results obtained with slices and homogenates of livers of rats and caviae arrive at the conclusion that the transfer of the methylgroup of methionine to guanidoacetic acid proceeds under influence of ATP.

The investigation on the synthesis of choline has shown that methionine is an active methyldonor. Methyldonors are not administered to the normally raised cultures of *Aspergillus niger*. It is therefore necessary that the fungus synthesizes the required methylgroups. Matchett, Marion and Kirkwood (99) have proved that sprouting barley synthesizes the methylgroup of choline from sodium formate.

Summary

1. Chapter I gives a survey of the literature on the metabolism of choline in plants and animals. The synthesis of choline appears to progress via methylation of ethanolamine. The methylgroups required in animals are mainly supplied by compounds with biologically labile methylgroups present in the ration. The existence of the synthesis

of methylgroups from compounds like formic acid is found only when special conditions prevail. In plants the methylgroups are synthesized from "one C-fragments". The synthesized choline can be built in (lecithin); during dissimilation it can be converted into trimethylamine and into betaine.

2. Chapter II proceeds to describe the methods of determining total, free, lipoid and watersoluble bound choline and betaine.

3. Chapter III mentions the results of a comparative investigation on the determination of choline according to 2 different methods. By the determinations of the choline fractions in plants it appeared, that in the higher plant the amount of total choline consisted mainly of lecithin and in the lower plant mainly of watersoluble bound choline. Lecithinase C present in the higher plant might give a wrong impression of the constitution of the choline fractions.

4. Chapter IV describes the dissimilation of choline in *Aspergillus niger:* the fungus appeared to oxidise added choline to betaine; oxidation under anaerobic conditions is small in comparison to oxidation under aerobic conditions. No relation between the dissimilation of choline and the metabolism of carbohydrate could be found.

5. Chapter V mentions the investigation of the synthesis of choline in *Aspergillus niger:* In the presence of carbohydrates a synthesis of choline from methionine and ethanolamine takes place. We have tried to obtain more data on the relation between the metabolism of carbohydrates and the synthesis of choline; Cantoni's supposition that the transfer of the methylgroup of methionine occurs by means of ATP is to be preferred to that of Barrenscheen, who supposes the transfer takes place after oxidation of methionine to methionine-sulfoxide.

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THE PODOSTEMACEAE OF THE NEW WORLD III

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I. Introduction

As has been stated in the introduction of the second part, this third part will include the remainder of the American part of the tribe Eupodostemeae of the subfamily Eupodostemoideae which was not treated in part I, viz. the genera Oserya, Devillea, Ceratolacis, Mniopsis, Podostemum and Castelnavia. Included are the dubious genera, and it also contains additions and corrections to part I, latin descriptions of new taxa, a list of collectors' numbers in this part, new references to the literature, and a general index to the third part.

The attention of the reader is drawn to a publication of Szafer (1952) in which a fossil Podostemacea from Europe has been described. As I have not seen the material it is at present impossible to judge the value of the discovery though it seems highly improbable that Podostemaceae ever lived in Europe.

The abbreviations of the literature are the same as in Part I and II and therefore it is referred to the list of references in part I. Some new ones are given in this part.

At the end of this study of the American Podostemaceae, a century after Tulasne's monograph of the whole family, I like to express my most sincere thanks to Prof. Bremekamp of Utrecht and Dr Bakhuizen van den Brink jr of Leiden, the former for his kind advice and the corrections of the English text, the latter for the Latin descriptions of the new taxa in Part III. Finally I wish to express my deepest appreciation to Dr G. Taylor of London for the discussions we had about this curious family, his warm interest for these studies and the final checking up of the text.

II. Taxonomic Section

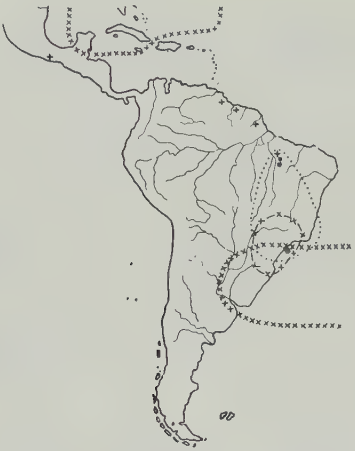
1. **OSERYA** Tul. & Wedd.

Small to very small herbs living in coenobia; the individuals opposite or subopposite along thin, branched roots, either thalloid or provided with a stem, the latter either hollow or solid and branched. Leaves often repeatedly forked, the forking extending over the whole leaf or confined to the top, sometimes with a distinct sheath and an intrapetiolar stipule, and sometimes indistinctly separated from the thalloid base. Flowers solitary; juvenile spathella clavate, fairly solid, mature one infundibuliform, mostly slightly exceeding the base; tepals 2 or 3, confined to one side, one at each side of the filament and usually a third one at its back; stamen 1, anther basifixed, dehiscing extrorsely to laterally, pollen grains ellipsoid to globose, 3-colpate; ovary 2-celled, with 2 unequal carpels and provided with 6–14 ribs, often obliquely inserted on the pedicel, and the sutures oblique and excentric; styles 2, very short, free or cohering. Fruit with two unequal valves, 6–14-ribbed, one of the valves sometimes caducous.
Type: *Oserya flabellifera* Tul. & Wedd.
Distr.: 6 species in Brazil, Guyana and Mexico. (See map)

This small genus is characterized by extrorsely dehiscing anthers. It was established by Tulasne and Weddell in 1852 with 4 species, one of which was *O. flabellifera*. Went (1910) believed that he had found this species in Suriname, and his study of the material on which this belief was founded led him to the conclusion that the genus could not be maintained and would have to be sunk in *Apinagia*. The above named species accordingly was named *A. flabellifera*. The Suriname material, however, proved to belong to a new species, viz. *O. minima*, which differs from the other ones in the presence of 3 filiform tepals and in the 8-ribbed ovary. The description of

O. sphaerocarpa matches that of this new species in most respects, but the material on which the latter is based, is so poor that the question of the identity of these species can not be answered.

Went was of opinion that the differential character of *Oserya*, viz. the extrorse dehiscence of the anthers is not sufficient to justify the separation from *Apinagia*. The present author admits that this difference is in itself insufficient, and he himself did not find it necessary to split *Jenmaniella* on account of the sometimes extrorsely and sometimes introrsely dehiscing anthers into two genera, confining himself to the distinction of two sections. Nevertheless he prefers to separate *Oserya* from *Apinagia* on account of the following additional characters: anthers basifixed in *Oserya*, dorsifixed in *Apinagia*; styles very short and knob-like in *Oserya*, longer and linear to subulate in *Apinagia*. The species of *Oserya* are always provided with a single stamen, and although this applies to some *Apinagia* species, it is in that genus far from general; moreover the *Oserya* species have often a tepal at the back of the only stamen, and this tepal is never met with in *Apinagia*. Taking all these characters together, it seems desirable to maintain *Oserya*. The genus is undoubtedly related to *Apinagia* but the insertion of the third tepal at the back of the single stamen points to an affinity with those genera which possess 2 stamens borne by an andropodium, where in the fork between the two stamens or, more often, slightly below this point, a third petal is always present.



DISTRIBUTION OF THE GENERA:
• Ceratolacis + Oserya
▪ Devillea ×-×-×- Mniopsis
××××× Podostemum ······Castelnavia

Geography (See map)

The genus is widely dispersed in Central America and the northern parts of South America. One species, viz. *O. coulteriana*, is found in the southern part of Mexico, and the other ones in Guiana and Northern Brazil, viz. *O. perpusilla* and *O. minima* in Suriname, *O. sphaerocarpa* in Demerara, and two, viz. *O. biceps* and *O. flabellifera* in Central Brazil. The distribution is rather remarkable, but it is possible that these species, which are easily overlooked on account of their small size, are present in the intervening parts too.

Key to the species:

1*a*. Plants with distinct, 1-3 cm long stems. Leaves repeatedly forked; ovary with 6 ribs; tepals 1-2 mm. Mexico . . . . 1. **O. coulteriana** Tul.
b. Plants thalloid or with a very short, 2-5 mm long, hollow stem. South America 2
2*a*. Plants thalloid 3
b. Plants with a short and hollow, 2-5 mm long stem. 5
3*a*. Leaves with a narrow, c. 0.5 mm wide petiole . 2. **O. minima** v. Royen
b. Petiole 1-10 mm wide 4
4*a*. Petiole c. 1 mm wide; pollen grains with numerous small warts . . . . 3. **O. sphaerocarpa** Tul.
b. Petiole 5-10 mm wide; pollen grains smooth . . . . 4. **O. perpusilla** (Went) v. Royen
5*a*. Flowers with 2 tepals; ovary with 12 or 14 ribs 6
b. Flowers with 3 tepals; ovary with 8 ribs . . . 2. **O. minima** v. Royen
6*a*. Fruit with 12 ribs . . . . 5. **O. biceps** Tul. & Wedd.
b. Fruit with 14 ribs . . . . 6. **O. flabellifera** Tul. & Wedd.

1. **Oserya coulteriana** Tul., (1849) 106; Tul. (1852) 153–156, t. 10 f. 2; Walpers (1852) 440; Weddell (1858) 787; Engler (1930) 64–Plate 1 f. 1-2.

Small coenobia consisting of 1–4 individuals clustered along a thin, strongly compressed root. Stems 1–3 cm × 1–2 mm, branched at the top, terete, at the base with a few rests of leaves, at the top with 1–12 cm long leaves, the latter a few times forked, ultimate divisions acute, 2–6 mm, very narrow; petiole 0.5–2 cm, terete, at the base usually provided with 2 wings; basal leaves without wings, and the uppermost leaves with a single, very short, obtuse, membranous wing. Flowers at the end of short branches, pedicel 2–5 mm; juvenile spathella clavate, obtuse to mucronate, slightly papillate, mature one infundibuliform, 4–12 mm; tepals 2, lanceolate, 1–2 mm, acute, one at either side of the filament and united with the latter; stamen 1.5–3.5 mm, anthers cordate, 0.8–1.5 mm, emarginate, base of thecae obtuse to mucronate, pollen grains 19.2 × 16 μ; ovary ellipsoid, 1.5–2.5 × c. 1 mm, obtuse, slightly attenuate at the base, subobliquely inserted on the pedicel, provided with 6 ribs; styles cylindrical, obtuse or with two short teeth, free, c. 0.5 mm. Fruit with 2 unequal valves, each valve with 3 distinct, prominulous ribs and 2 indistinct marginal ones.
Type: Coulter 1394 in K.
Distr.: Mexico.

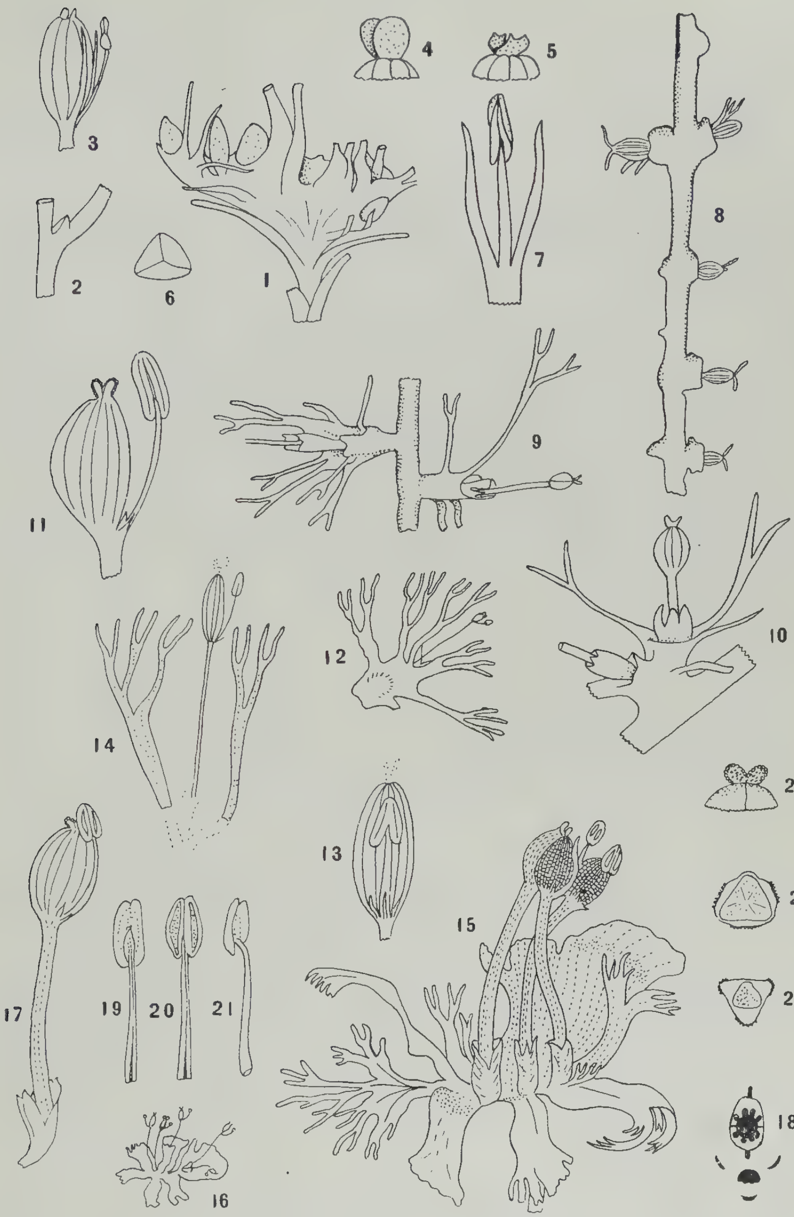


PLATE 1 — 1-7. **Oserya coulteriana** Tul. *(Arsène 5255)*, 1. habit (leafless); 2. stipule and leaf-base; 3. flower; 4-5. styles; 6. pollen grains; 7. androecium — 8-11. **Oserya minima** v. Royen *(Went s.n.)*, 8. root with secondary shoots; 9. root with two shoots with leaves; 10. one shoot; 11. flower diagram — 12-14. **Oserya sphaerocarpa** Tul. & Wedd. *(Schomburgk 431)*, 12. habit; 13. flower; 14. flower and leaves — 15-24. **Oserya perpusilla** (Went) v. Royen *(Lanjouw 723)*, 15-16. habit; 17. flower with opened spathella; 18. flower-diagram; 19-21. stamen from the inner, outer and lateral side; 22. styles; 23-24. pollen grains.

Prov. Jalisco, near San Blas, Coulter 1394, fl. fr. (GH, K, NY, P); *prov. Michoacan*, near Rincón, Arsène 5255, fl. fr. Oct. (GH, MO); near San Miguel, Arsène 5965, fl. fr. Nov. (GH, MO).

2. **Oserya minima** v. Royen, nov. sp. – P. 258 and plate 1 f. 8-11.

Very small coenobia, the individuals subopposite to opposite along thin, branched, compressed roots. Base thalloid or tubuliform, up to 4 mm high. Leaves a few times forked, up to 5 mm, petiole c. 0.5 mm wide; ultimate divisions subfiliform. Flowers solitary; pedicel c. 1 mm; juvenile spathella clavate, obtuse, sessile, stout, slightly exceeding the thalloid base, mature one tubuliform, c. 1.5 mm; tepals 2 or 3, c. 0.3 mm, one at either side and one at the back of the filament, sometimes one of the lateral sepals absent; stamen 1–1.3 mm, anthers cordate, c. 0.5 mm, obtuse or emarginate, thecae sometimes unequal, base of thecae obtuse, pollen grains 14.8 × 10.7 μ; ovary obliquely ellipsoid to globose, c. 1.5 × 1 mm, obtuse, attenuate at the base, with 2 unequal carpels and 8-ribbed; styles short, c. 0.3 mm, subpapillate, cylindrical, obtuse, cohering at the base. Mature fruit unknown, but according to WENT the smaller valve caducous.
Type: Went s.n. in U.
Distr.: Suriname.

Suriname-river, near Kabelstation, Went s.n., fl. Sept. (U), cited as *Apinagia flabellifera* (non Tul. & Wedd.) in Went, (1926) 36-37, t. 2 f. 12, t. 6 f. 37-38.

3. **Oserya sphaerocarpa** Tul. & Wedd., Tul. (1849) 106; Tul. (1852) 154–155; Walpers (1852) 440; idem (1858) 787; Weddell (1873) 84; Baillon (1888) 272—Plate 1 f. 12-14.

Small thalloid coenobia, up to 5 mm long and wide. Leaves at the top a few times forked, widened at the base and provided with two wings, c. 1 mm long, petiole c. 1 mm wide. Flowers solitary; pedicel 6–8 mm; spathella unknown; tepals 3, very short, filiform, acute, less than 0.5 mm long; stamen c. 1.5 mm, anther cordate, c. 0.5 mm, obtuse, base of thecae obtuse or subacute, pollen grains of two types, one type ellipsoid, 3-colpate, the other one globose with numerous warts, 15.5 × 10.6 μ; ovary ellipsoid, c. 1 mm high, obtuse, rounded at the base, with two unequal carpels and provided with 12 ribs, sutures excentric, oblique; styles unknown. Fruit with one caducous valve, both valves with 7 ribs; pedicel 1 cm.
Type: Schomburgk in K.
Distr.: British Guiana.

Bernice river, Schomburgk 431, fl. fr. (C, K, P, W).

4. **Oserya perpusilla** (Went) v. Royen, nov. comb.—*Apinagia perpusilla* Went in Pulle (1909) 267; Went (1910) 43–46, t. 12 f. 106–114; Engler (1930) 39; v. Royen (1948) 382—Plate 1 f. 15-24.

Small coenobia with a narrow base, up to 3 cm large. Leaves cuneate, at the top a few times forked, 1–2 cm, indistinctly nerved,

petiole 5–10 mm wide. Flowers white; pedicel 4–14 mm; tepals 2 or 3, subulate, 0.5 mm long or less, one at either side of the filament and one at the back of the latter, but this one sometimes absent; stamens 1–2 mm, anther cordate, up to 1 mm, obtuse or emarginate, pollen grains 15.6 × 12.7 μ; ovary ellipsoid, 1–2 × 0.5–1 mm, obtuse, terete, rounded at the base or subattenuate, with two unequal carpels and provided with 12 ribs, sutures excentric; styles up to 0.5 mm, free, strongly papillate. Fruit with 2 unequal valves, each valve with 7 or 5 ribs, i.e. the marginal ones sometimes very indistinct or absent, one valve caducous.
Type: Versteeg 810 in U.
Distr.: Suriname and French Guiana.

Suriname: Tapanahony river, Kortufutu-falls, Versteeg 810, fl. fr. Aug. (P, U); Marowyne-river, Armina-falls, Went s.n., fl. fr. Oct. (P, U); Coppename-river, Raleigh-falls, Went s.n., fl. fr. Aug. (U); idem, Lanjouw 723, fl. fr. Sept. (U); Saramacca river, Grand Dam, Maguire 24919, fl. fr. Oct. (F, NY, U, US).
French Guiana: Rio Oyapock, Salto Caxiry, v. Luetzelburg 21655, fl. March (M); idem, Grande Roche, v. Luetzelburg 21691, fl. July (M).

5. **Oserya biceps** Tul. & Wedd., Tul. (1849) 106; Tul. (1852) 152–153; Walpers (1852) 440; Tul. (1863) 260–261; Walpers (1858) 787; Weddell (1873) 83–84; Engler (1930) 64.

Small coenobia with a more or less distinct stem, 3–5 mm. Leaves 2–3 mm, linear, sheathed at the base. Flowers solitary; pedicel 3–5 mm; mature spathella tubuliform; tepals 2, c. 0.3 mm, one at either side of the stamen, linear; anther c. 1 mm, obtuse; ovary ovoid-ellipsoid, 1.5 × 1 mm, obtuse, rounded at the base, provided with 12 ribs, styles unknown. Fruit with two persisting valves (The description is taken from TULASNE, as I have not seen the material).
Type: Weddell s.n. in P (?).
Distr.: Brazil (Province Goyaz)

Rio Araguay, Weddell s.n., fl. fr. (P)?. WEDDEL gives the Rio Tocantin as the type locality.

6. **Oserya flabellifera** Tul. & Wedd., Tul. (1849) 106; Tul. (1852) 151–152, t. 10 f. 3; Walpers (1852) 440; Walpers (1858) 787; Tul. (1863) 259–260, t. 75 f. 3; Weddell (1873) 83; Warming (EP 1891); Engler (1930) 64, f. 56 A—Plate 2 f. 1-3.

Roots, stem and leaves similar to those of O. minima. Flowers with 2 spatulate, c. 0.3 mm long tepals, one at either side of the filament; anther subcordate, c. 0.5 mm, obtuse, base of thecae acute, pollen grains 20 × 19 μ; ovary ellipsoid, subacute, with unequal carpels and provided with 14 lighter coloured streaks, in other respects similar to O. minima, styles lanceolate, 3-sided, otherwise similar to O. minima. Fruit unknown.
Type: Weddell s.n. in P.
Distr.: Brazil (Province Goyaz)

Rio Tocantin, fl. (C, P).

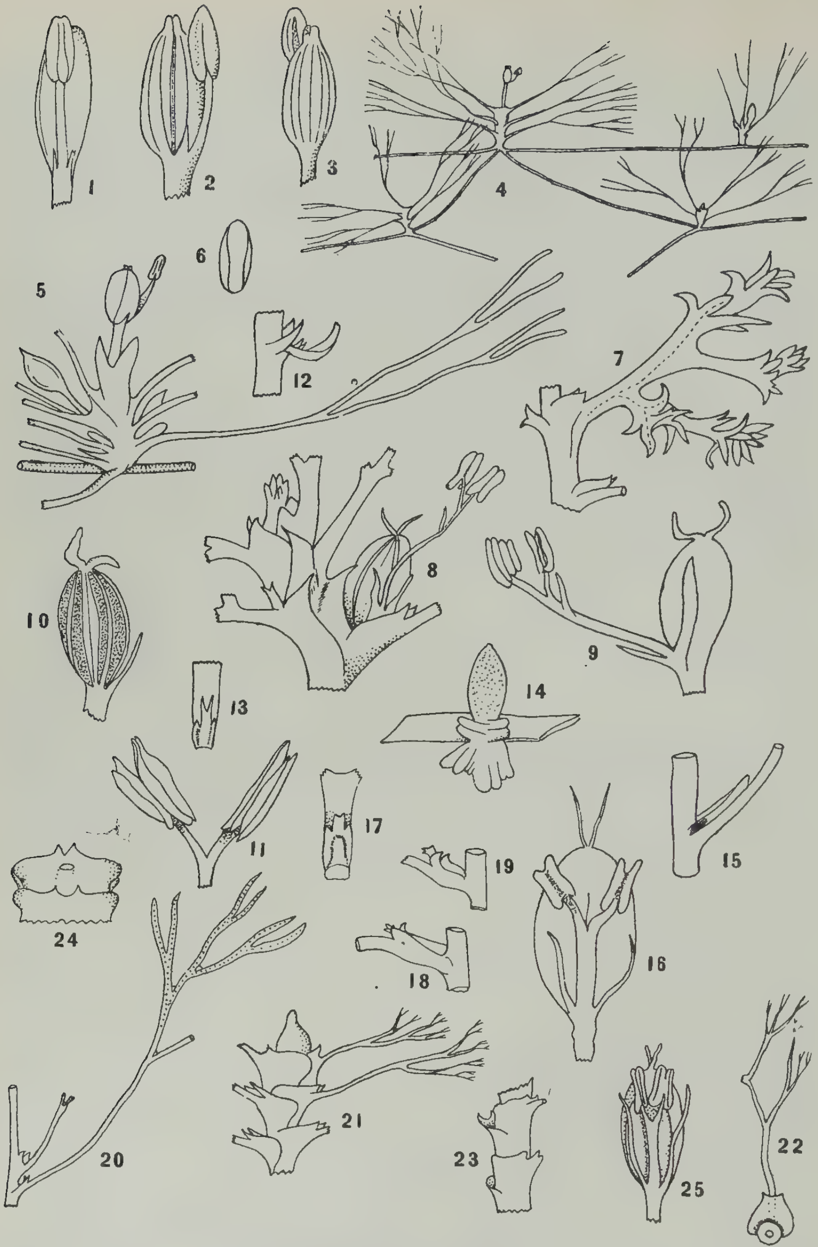


PLATE 2 — 1-3. **Oserya flabellifera** Tul. & Wedd. *(Weddell s.n.)*, 1-3. different views of the flower — 4-6. **Devillea flagelliformis** Tul. & Wedd. *(Weddell 2367)*, 4. part of coenobium; 5. secondary shoot; 6. pollen grain — 7-13. **Podostemum distichum** (v. Cham.) Wedd. *(Sellow s.n.)*, 7. leaf; 8. apical part of a secondary shoot; 9. flower; 10. fruit; 11. stamen; 12-13. stipules — 14-16. **Podostemum dimorphum** v. Royen *(Dusén 16540)*, 14. fertile plant showing the haptere; 15. stipule; 16. flower — 17-19. **Podostemum schenckii** Warming *(Schenck 328)*, 17-19. different types of stipules — 20. **Podostemum undulatum** v. Royen *(Ule 804)*, habit — 21-25. **Podostemum glaziovianum** Warming *(Glaziou 21993)*, 21. top of a shoot; 22. leaf with stipule from above; 23. basal part of a shoot; 24. stipule, frontal view; 25. flower.

2. DEVILLEA Tul. & Wedd.

Small unbranched individuals springing in groups of 1 to 4 from branched, filiform roots. Stem at the base with few rests of leaves, at the top with entire or, sometimes, forked leaves, base of the leaves strongly amplexicaul. Flowers few, within a clavate to globose, acuminate spathella, which is enveloped by two membranes formed by the sheaths of the apical leaves; tepals 3, linear, one on either side of the filament and one at the back of the latter; stamen 1, anther obtuse, dehiscing introrsely, pollen grains ellipsoid, 3-colpate; ovary ellipsoid to globose, with 2 unequal carpels, sometimes with an excentric suture, placenta ellipsoid, strongly compressed; styles very short, cohering at the base, slightly papillate. Fruit with 2 unequal valves, one of the valves caducous.
Type: *Devillea flagelliformis* Tul. & Wedd.
Distr.: One species in Central Brazil. (See map)

This genus was founded by TULASNE and WEDDELL. BAILLON, however, sunk it in *Oserya*, and WARMING (1891) and ENGLER (1930) followed his example. To the present author this seems incorrect, as too many differences exist between the two genera: the anthers dehisce introrsely (against extrorsely in *Oserya*), and the individuals are placed either in groups of 2 to 4 or solitary (in *Oserya* they are opposite or subopposite along the roots, except *Oserya coulteriana* where, however, the ovary is ribbed). These three differentiating characters are of sufficient importance to maintain *Devillea* as a separate genus.

1. **Devillea flagelliformis** Tul. & Wedd., Tul. (1849) 107; Tul. (1852) 157–159, t. 13 f. 1; Walpers (1852) 440; Walpers (1858) 788; Tul. (1863) 262–263, t. 75 f. 4; Weddell (1873) 82; Bentham & Hooker (1880) 114—*Oserya flagelliformis* (Tul. & Wedd.) Baillon, (1888) 272; Warming (EP 1891) 21—*Oserya flagelliformis* (Tul. & Wedd.) Warming, Engler (1930) 64, f. 56 B–H—Plate 2 f. 4-6.

Stems subcompressed, 0.5–1 mm. Leaves 2–2.5 cm, repeatedly forked, intrapetiolar stipule large, linguiform, membranous, c. 0.5 mm; petiole triangular, 5–10 mm, ultimate divisions subfiliform, obtuse. Pedicel 0.5–1 mm; mature spathella widely campanuliform, up to 1 cm; tepals lanceolate, obtuse, up to 0.5 mm; stamen 1–1.5 mm, anther 0.5–0.8 mm, obtuse, pollen grains 20.1 × 13.2 μ; ovary 1–1.5 × c. 1 mm, styles cylindrical, obtuse, up to 0.3 mm.
Type: Weddell 2367 in P.
Distr.: Brazil (Province Goyaz).

Rio Tocantin near Sao João, Weddell 2367, fl. fr. July (C, P).

3. CERATOLACIS (Tul.) Wedd.

Very small individuals provided with a small stem or stemless and inserted along vermiform, branched roots. Leaves either entire and

linear or a few times forked with the ultimate divisions linear. Flowers few to one; juvenile spathella clavate, obtuse; tepals 2 or 3, one on either side of the andropodium, the third in the fork between the two filaments; stamens 2, borne by an andropodium; anther dehiscing introrsely; ovary fusiform, with 6 prominent ribs; midribs of the carpels running out into the styles, carpels equal; styles rostriform, when young deflexed towards the anthers. Fruit similar to the ovary, with two persisting valves.
Type: *Ceratolacis erythrolichen* (Tul. & Wedd.) Weddell
Distr.: One species in Central Brazil. (See map)

1. **Ceratolacis erythrolichen** (Tul. & Wedd.) Wedd., (1873) 66; Bentham & Hooker (1880) 113; Baillon (1888) 263; Warming (EP 1891) 21; Engler (1930) 52, f. 43 A–E—*Dicraea erythrolichen* Tul. & Wedd., Tul. (1849) 102; Tul. (1852) 126–128, t. 10 f. 1–5; Walpers (1852) 437; Walpers (1858) 783; Tul. (1863) 253–254, t. 74 f. 1.

Roots fleshy, 5–10 × 1–2 mm. Leaves distichous, 2–3 mm, a few times forked or entire. Flowers solitary or few; pedicel up to 3 mm; tepals filiform; stamens 2; anther dorsifixed, elliptic, top retuse; ovary fusiform, with 6 prominent ribs. (The description is partly extracted from Tulasne (1852), as the material was too incomplete to provide sufficient details.)
Type: Weddell s.n. in P.
Distr.: Brazil. (Province Goyaz).
Rio Tocantin, Weddell s.n., fl. fr. July (K, M, P).

4. **MNIOPSIS** Mart. & Zucc.

Coenobia consisting of small individuals, opposite or subopposite along branched roots, stems distinct, branched or unbranched, mostly with rests of leaves on the basal part and a tuft of leaves at the top. Leaves either all similar and provided with one or two intrapetiolar stipules, or of two types and then estipulate and in 4 rows. Leaves and stipules obliquely inserted, with the apical end of the insertion area nearest to the substratum. Leaves entire or a few times forked. Flowers few to numerous; pedicel mostly short, slightly exceeding the spathella; juvenile spathella nipple-shaped; tepals 3 or 2, one on either side of the andropodium, the third in the forking between the two stamens and sometimes absent; stamens borne by an andropodium, anthers dehiscing introrsely, pollen grains 2-celled; ovary globose to ellipsoid, with two unequal cells, sutures oblique; styles simple or branched, mostly densely papillate. Fruit similar to the ovary, the smaller valve caducous.
Type: *Mniopsis scaturiginum* Mart. & Zucc.
Distr.: 5 species in SE Brazil. (See map)

This genus is easily recognizable on account of its smooth ovary and the leaves provided with a distinct stipule which is rarely absent in some specimens of *M. crulsiana*. The branched styles in some species often give a good indication to which genus a species belongs. It was founded by Martius and Zuccarini in 1824 and Warming between 1881 and 1888 added some more species.

M. weddelliana has the largest distribution, for its range covers the Brazilian provinces Rio de Janeiro, Sao Paulo, Minas Geraes, Rio Grande do Sul and Santha Catharina. It proves to be highly variable, and attempts have been made by Warming to distinguish a number of forms, but on account of the numerous intermediate stages this seems to be impossible.

Key to the species:

1*a*. Leaves entire or with 2-lobed top, 2 mm long or less . . . . 1. **M. scaturiginum** Mart & Zucc.
b. Leaves a few times forked, up to 2.5 cm long 2
2*a*. Styles entire; leaves in 4 distinct orthostichies, estipulate . . . . 2. **M. glazioviana** Warming
b. Styles branched; leaves in 2 orthostichies and provided with stipules 3
3*a*. Ultimate divisions spatulate to ovate . . . . 3. **M. weddelliana** Tul.
b. Ultimate divisions filiform 4
4*a*. Stipules acute, distinct; leaves 1-2.5 cm long . . . . 4. **M. saldanhana** Warming
b. Stipules obtuse, indistinct or absent; leaves 0.2-10 cm long . . . . 5. **M. crulsiana** Warming

1. **Mniopsis scaturiginum** Mart. & Zucc., (1824) 3–4, t. 1; Schnitzlein (1843–1846) t. 85 f. 5; Tul. (1849) 105; Tul. (1852) 143–145; Walpers (1852) 439; Walpers (1858) 786; Tul. (1863) 257–258; Weddell (1873) 77; Warming (EP 1891) 21; idem (1899) 135–138, t. 31 & 32; Glaziou (1911) 576; Engler (1930) 60, f. 52 A–E; Tobler (1933) 298–299, f. 15—*Crenias scopulorum* Sprengel, (1827) 247.

Small coenobia, forming dense mats, stem 2–4 cm, branched. Leaves in 4 orthostichies, the largest leaves in the outer orthostichies, the two other orthostichies with smaller leaves. The larger leaves subrotundate, up to 2 × 2.5 mm, top truncate or in the apical ones 2-lobed, base widened, decurrent, the smaller leaves up to 1 × 0.5 mm, top rounded, base widened, decurrent, both types of leaves coriaceous, entire, and nerveless. Flowers terminal; pedicel up to 5 mm; juvenile spathella nipple-shaped, dehiscing laterally, mature spathella campanuliform, up to 3 mm; tepals 3, two of them linear-lanceolate, up to 2 mm, the third one filiform, c. 1 mm, andropodium membranous, up to 1.5 mm, filaments up to 0.5 mm, anthers 4-sided, up to 1.5 mm, the 2 outer loculi larger than the inner ones, top and base of the thecae emarginate, pollen grains unknown; ovary globose, up to 2 mm diam, styles markedly papillate, up to 0.5 mm; each divided in 3 or 4 cylindrical branches. The smaller valve of the fruit caducous.
Type :Martius s.n. in M.
Distr.: Brazil. (Provinces Goyaz, Matto Grosso, Minas Geraes, Santha Catharina)

Goyaz — near Contagem de S. Maria, Martius s.n., fl. fr. Oct. (BM, L, M, P, W); Rio Babylon, Glaziou 21985, fl. fr. Aug. (NY); Rio Trinidade, Glaziou 21994, fl. fr. Aug. (C, G-Del., P); Rio Carumba, Glaziou 21997, fr. July (BR, C, G-Del., P), idem, Glaziou 21998, fr. July (BR, C, G-Del., P, S); Rio Uruhu, between Jaraguay and Goyaz, Glaziou 22003, fl. fr. July (BR, C, F, G-Boiss., P, S, US); Rio Macaco, Glaziou 22004, July (BR, C, F, G-Boiss., K, P, S); Rio de Almas, near Meia Ponte, Glaziou 22008, fr. Aug. (BM, BR, C, G-Del., P, S, US); idem, Glaziou 22009, fl. fr. Aug. (BM, BR, C, F, G-Del., P, S, US); idem, Glaziou 22010, Aug. (BM, BR, C, P, S); idem, Glaziou 22011, fl. fr. Aug. (BR, C, F, G-Del., P, S, US); Ribeirão de Caldas, Glaziou 22013, fl. fr. July (BM, BR, C, F, G-Boiss., G-Del., P, S, US); Rio Vermelho, Weddell s.n. (P) — *Matto Grosso* — without loc., Moore 711 (BM) — *Minas Geraes* — Caldas, Regnell s.n., Aug. (BR) — *Santha Catharina* — Rio Humboldt, Ehrhardt s.n., fl. Oct. (BM) — *Unknown prov.* — Serra de Carawi, Mosén 359, Aug. (S) — *Without loc.* — Vauthier s.n., (W); Burckell 5665, (W).

2. **Mniopsis glazioviana** Warming, (1881) 1–26, t. 6 f. 9–15; idem (1882) 118–120, t. 9 f. 37–53; idem (1888) 481; idem (1899) 138; Glaziou (1911) 575–576; Engler (1930) 60, f. 6, 20 F; Kuhlmann & Kühn (1947) 15, 54, f. 5.

Small herb with a slightly branched stem, which is up to 1 cm long and c. 0.5 mm diam, quadrangular on account of the decurrent margins of the leaves. Leaves up to 2 cm, base ovate, decurrent, provided with a triangular, acute, up to 1 mm long stipule, which is subamplexicaul and decurrent, top a few times forked, the lower leaves often without forked top. Flowers few; pedicel up to 1 cm; juvenile spathella nipple-shaped, mature one infundibuliform, up to 3 mm; tepals 3, the two lateral ones linear, up to 1 mm, the third one shorter and narrower, andropodium flattened, up to 1.5 mm, filaments up to 0.5 mm, anthers quadrangular, up to 0.8 mm, top and base emarginate, inner loculi shorter than the outer ones, thecae unequal, emarginate at either end, pollen grains unknown; ovary subglobose to ellipsoid, up to 1.5 mm, styles filiform, entire, more or less cohering at the base, up to 0.5 mm, markedly papillate. Fruit similar to the ovary, the smaller valve caducous.
Type: Glaziou 12191 in C.
Distr.: Brazil. (Provinces Sao Paulo, Rio de Janeiro, Santha Catharina)

Rio de Janeiro — Rio Soberbo, Glaziou 12191, fl. fr. Aug. (BR, C, G-Del., P); idem, Glaziou 12193, March (BR, C, G-Del, GH, NY, P); Rio Bengala, Glaziou 12197, fr. Oct. (C, P); Rio Parahyba, near Boa Vista, Glaziou 13144, fr. July (C, P); Rio Soberbo, near Sition de H. Dias, Glaziou s.n., March (P) — *São Paulo* — Rio Camanducara, Cachoeira de Falcão, Kuhlmann & Kühn 970, Aug. (ex litt.) — *Santha Catharina* — Rio Humboldt, Ehrhardt s.n., fl. Oct. (BM); Rio Batatas, Riedel s.n., (C, LE-I) — *Without any details* — Schwacke 3298 (C).

3. **Mniopsis weddelliana** Tul. (1849) 105; idem (1852) 145–147, t. 8 f. 4; Walpers (1852) 439; idem (1858) 786; Tul. (1863) 258, t. 74 f. 4; Weddell (1873) 77; Warming (1881) 1–26, t. 4 f. 14–24, t. 5 f. 1–24, t. 6 f. 1–8; idem (1891) t. 8 f. 1–34, t. 9 f. 1–36; idem (1899) 138; idem (EP 1891) 21; Glaziou (1911) 576; Engler (1930) 60.

var. **weddelliana**

Small herb with a branched or unbranched, 0.5–3 cm high stem. Leaves a few times forked, flabelliform, 0.5–2.5 cm, ultimate divisions spatulate to ovate, 2–3 × 0.5–1 mm, obtuse to acute, with a single distinct nerve, petiole 1–3 mm, widened at the base, subamplexicaul, at the lowest margin provided with a triangular, acute, 1–1.5 mm long stipule; basal leaves often squamiform, subamplexicaul, acute, 0.5–1 mm. Flowers solitary; pedicel 0.5–2 mm; juvenile spathella clavate, nipple-shaped; mature one campanuliform, 2–2.5 mm; tepals 3, filiform, one on either side of the andropodium, 1.5–2 mm, the third one inserted in or slightly below the point of fusion of the filaments, 1 mm long or less, filaments 1–1.5 mm, anthers sagittate, 1–1.5 mm, top truncate or emarginate, base deeple incised, pollen grains 2-celled or, sometimes 3- or 4-celled, 41.6 × 24.8 μ; ovary obliquely ellipsoid, 1–1.5 mm, styles provided with 4–6 branches, 0.5–1 mm, strongly papillate. Fruit similar to the ovary, the smaller valve caducous.
Type: Weddell s.n. in P.
Distr.: Brazil. (Provinces Rio de Janeiro, Sao Paulo, Minas Geraes, Rio Grande do Sul, Santha Catharina)

Rio de Janeiro — Piabanha-river, Weddell s.n., fl. fr. Nov. (P); Serra des Orgnes, Petropolis near San Antonio, Glaziou 7398, fr. July (G-Del., P); Rio Bengala, between Nova Friburgo and Alto, Glaziou 12197, Oct. (C); Rio Itamaraty, near Petropolis, Glaziou 12198, May (C, P); without loc., Glaziou 8888 (BR); Rio Paqueques, dos Nevès Armond s.n., (C) — *São Paulo* — Cachoeira de Tres Pontes, Harshberger 977, May (US); Tres Pontes, Amparo, Hoehne s.n., fl. fr. May (SP); Salto de Piracicaba, Accorsi s.n., fl. Nov. (PIR); idem, Accorsi s.n., fl. fr. Aug. (PIR). *The two last-named specimens were mentioned by* ACCORSI (1944) 93-104, f. 13-17 *and* ACCORSI (1946) 400-423, f. 19, 24, 25, 27, *under the name Mniopsis glazioviana* Warming — *Minaes Geraes* — Pogos near Caldas, Regnell s.n., fl. Aug. (BR, C); Caldas, Regnell III 1117, June (C); Caldas, Regnell III 1118, fl. fr. March (C, S, US); Caldas, Regnell 1118 B & C, fl. fr. March (S); Caldas, Mosén 749, fr. (S) — *Rio Grande do Sul* — Rio Burges, without coll., March and April (P) — *Santha Catharina* — Rio Humboldt, Ehrhardt s.n., fl. fr. Oct. (BM) — *Without known province* — Rio Avayandaba, Riedel 395, (C) — *Without loc.* — Riedel 47, fl. fr. (C).

var. **gracilis** Warming, (1899) 138.

This variety differs from the var. weddelliana in the longer and narrower leaves, the acute ultimate divisions, and the pedicel up to 2.5 cm. As no flowering specimens were at hand, I have not been able to decide whether the specimens referred to, represent a variety of the species mentioned above or whether they are, as WARMING suggests, to be regarded as a distinct species.
Type: Glaziou 17777 in C.
Distr.: Brazil. (Province Rio de Janeiro)

Rio Grande, near Nova Friburgo, Glaziou 17777, Dec. (C, P); Rio Soberbo, Glaziou 12192, March (BR, C, F, G-Del., P, US); Rio Piabanha, near Petropolis, Glaziou 12194, May (BR, C, F, G-Del., US).

4. **Mniopsis saldanhana** Warming, (1888) 458–460, 481, t. 20 f. 10–29; Glaziou (1911) 575; Engler (1930) 60, f. 2 & 20 A–E.

Small herb with a stem up to 5 mm, strongly branched at the top, branches congested. Leaves a few times forked, 1–2.5 cm, ultimate divisions subfiliform; base subamplexicaul, 2–5 mm, widened, decurrent, provided with a single lanceolate, 1.5–2 mm, acute, stipule. Flowers relatively numerous, slightly protruding beyond the spathella; pedicel very short; juvenile spathella clavate, umbonate, mature one infundibuliform, up to 2.5 mm; tepals 3, one on either side of the andropodium, linear, 1–1.5 mm, the third one linear, up to 0.8 mm, andropodium up to 2.5 mm, filaments 0.5 mm, anthers quadrangular, top and base deeply incised, loculi inserted at different levels, pollen grains 2-celled, 46.5 × 27.9 μ; ovary ellipsoid, 1.5–2 × 1–1.5 mm, sutures distinct, oblique, styles up to 1 mm, strongly papillate, provided with 6–9 branches. Fruit incompletely known.
Type: Glaziou 13146 in C.
Distr.: Brazil (Province Rio de Janeiro)

Rio Bengala near Nova Friburgo, Glaziou 13146, Febr. (BR, C, F, G-Del. P, US); idem, Glaziou 17225, Dec. (BM, BR, C); idem, Glaziou 17229, fl. (P).

5. **Mniopsis crulsiana** Warming, (1899) 138–140, f. 33–34; Engler (1930) 60, f. 52 G.

Coenobia consisting of small individuals arising from 0.2 mm wide, flattened roots, and either stemless or provided with a short, i.e. not more than 12 mm high, stem. Leaves a few times forked, 2–10 mm, ultimate divisions subfiliform, up to 1 mm, base widened, subamplexicaul, provided with a single stipule, appearing in the form of a narrow, obtuse crest, or without stipule; petiole relatively long. Flowers few; juvenile spathella clavate, mature one narrowly infundibuliform; tepals 3, linear, two of them reaching ¾ of the length of the andropodium, the third one slightly shorter, anthers quadrangular, top emarginate. pollen grains 2-celled, incompletely known; ovary globose, styles provided with 4–6 branches; densely papillate. Fruit unknown. (The description of the flower is extracted from the publication of WARMING)
Type: Glaziou s.n. in C.
Distr.: Brazil.

Rio Alto Macahé, Glaziou s.n., fl. June (C, U).

DUBIOUS SPECIES

1. **Mniopsis guianensis** Klotsch, Schomburgk (1848) 930.

This species is reported from the Essequibo, Mazaruni and Cuyuni rivers but as no specimens are indicated by KLOTSCH I was unable to trace this species.

5. PODOSTEMUM Michaux

Small to medium-sized coenobia arising from the flanks of branched roots; individuals provided with a more or less distinct stem or stemless, branched or unbranched. Fertile individuals sometimes separate from the sterile individuals along the same root. Leaves distichous, repeatedly forked or entire, sometimes obliquely inserted, provided with a distinct intrapetiolar stipule, in the lower leaves the peripheral parts nearly always suppressed and the stipule reduced or lacking. Flowers axillary, spathella splitting at the top; pedicel as a rule relatively short; tepals 3; one one either side of the andropodium and the third one in the fork between the two filaments; stamens 2, borne by an andropodium, filaments rather short, anthers dehiscing introrsely, pollen grains 2-celled; ovary consisting of two subequal cells and provided with 6 or 8 ribs, styles simple, equal or unequal, free. Fruit similar to the ovary, the smaller valve caducous.

Type: *Podostemum ceratophyllum* Michaux

Distr.: 17 species in southern and southeastern parts of North America, in the West Indian islands, Central America, southern and south-western parts of Brazil, Uruguay, Paraguay, Argentina, tropical parts of Africa, Ceylon, India. (See map)

Taxonomy

The genus was founded in 1803 with a single species, viz. *P. ceratophyllum*. The correct form of the name of this genus and consequently that of the name of the family and of the order, has for a long time been a subject of dispute. In A. MICHAUX's 'Flora boreale-americana' the plant on which the genus was based is described under the name *Podostemum ceratophyllum*, but in the explanation of the accompanying figure it is named *Podostemon ceratophyllum*. SPRAGUE discussed this question in 1933 and came to the conclusion that the form *Podostemum* must be regarded as the right one. The discovery of the type specimen in the Paris herbarium proved the correctness of this interpretation as that sheet bears the name *Podostemum ceratophyllum* in the handwriting of A. MICHAUX.

As WARMING had accepted the form *Podostemon*, his new species were all published under this generic name. ENGLER (1930) too regards *Podostemon* as the correct form, and he introduced therefore for the order the name *Podostemonales*, a name also erroneously used by the editor of the Bulletin of the Missouri Botanical Garden in a publication of the present author in 1950.

In the course of the present study it became necessary to describe some new taxa, viz. *P. ceratophyllum* var. *circumvallatum*, *P. dentatum*, *P. dimorphum*, *P. undulatum*, with the var. *angustifolium*, while one new combination had to be made, viz. *P. ricciiforme*. *P. warmingii* proved to be conspecific with *P. aguirense*.

P. ceratophyllum var. *circumvallatum* differs from the type in the shorter stems, the densely congested bases of the leaves, and the larger number of rests of leaves on the basal parts of the stem. The flower unfortunately is unknown. The varieties *abrotanoides* (Nuttall) Weddell and *chondroides* Fassett can not be maintained as they are merely extreme variants connected with the ordinary form by a series of intermediate stages. *P. dentatum* van Royen, *P. mülleri* Warming, *P. uruguayense* Warming, and *P. galvone* Warming differ from the other species of this genus in the stipule which is inserted with a narrow, linear base on the petiole. The other species have a stipule which is inserted with a wide and concave base. *P. dentatum* resembles *P. galvone* in the presence of teeth along the upper part of the petiole, but differs in the latter's sudden contraction towards the top, in the greater length of the stipule, and also in its small size.

P. dimorphum is remarkable on account of the disproportion between the large sterile stems and the minute, fertile ones, which bear but one flower. In its stipule it closely resembles *P. ceratophyllum.* The leaves resemble those of *P. ceratophyllum* but have wider and longer ultimate divisions.

P. undulatum also resembles *P. ceratophyllum*, and *P. aguirense* as well, in its leaves and stipules, but differs in the spathulate and longer ultimate divisions. It also resembles *P. schenckii* and *P. ostenianum* but differs from the first in the abrupt widening of the ultimate divisions and from the latter in the greater width of the ultimate divisions, which, moreover, are provided with a distinct nerve. The variety *angustifolium* has a longer stem, narrower ultimate divisions and longer stipules.

Marathrum? *ricciaeforme* is on account of its stipulate petiole transferred to *Podostemum*, but as long as its flowers are unknown, its true position remains uncertain.

The species of this genus are difficult to define. Floral details are useless, as the floral parts differ only in their dimensions and as these differences moreover are too small to be used as distinctive characters. More serviceable differences are found in the leaves and in the stipules.

Geography (See map)

The distribution of this genus is anomalous. One part of its area covers the southern and eastern parts of North America, the West Indian islands and the eastern parts of Central America; the other and greater part extends from Central Brazil southwards to Uruguay, Argentina and Paraguay. Some species are reported from Africa but it is doubted whether these really belong to this genus. *P. ceratophyllum*, whose range extends from eastern North America over the West Indian islands to the Yucatan Peninsula in Central America, has the widest distribution. The most northern habitat is in the neighbourhood of Quebec.

Key to the species [1,2]

1*a*. Ultimate divisions of the leaf consisting of 2-5 irregularly whorled, triangular, up to 2.5 mm long segments . . . 1. **P. distichum** (v. Cham) Wedd.
b. Ultimate divisions linear or spatulate to filiform, not whorled 2
2*a*. Leaves entire 3
b. Leaves repeatedly forked 6
3*a*. Upper part of leaf-base provided with a single marginal tooth 4
b. Such a tooth not present 5
4*a*. Leaf-base gradually passing into the wider limb. 15. **P. galvone** Warming

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[1] The characters of stipules and leaves are those observed in the uppermost leaves.
[2] **Podostemum comatum** Hicken is not included in the key (See p. 244).

b. Leaf-base abruptly narrowed before passing into the limb . . . . 13. **P. dentatum** van Royen

5*a.* Stipule inserted either near the top or slightly shifted towards the middle of the leaf-base, triangular to trapezoid, acute. Leaf-bases numerous, spatulate, 2-5 × 1-2 mm, flaccid, sometimes provided with an about 3 cm long filiform petiole and a forked upper part with spatulate ultimate divisions . . . . 16. **P. mülleri** Warming

b. Stipule in the basal leaf-bases inserted in the middle of the leaf-base, quadrangular to ovate, acute to obtuse. Leaf-bases few, irregularly trapezoid, acute, 4-8 × 2-5 mm, coarse, sometimes keeled above . . . . 14. **P. uruguayense** Warming

6*a.* Leaves with a single stipule 8

b. Leaves with two marginal stipules 7

7*a.* Ultimate divisions subfiliform to linear, 2-4 mm long; stipules obtuse, inconspicuous . . . . . 12. **P. fruticulosum** (Tul. & Wedd.) Wedd.

b. Ultimate divisions spatulate, about 1 × 0.5 mm; stipules acute, well developed . . . . 9. **P. ostenianum** Warming

8*a.* Stipules inserted with a wide, concave, base, boat-shaped 9

b. Stipules inserted with a laterally flattened base, crest-like 27

9*a.* Stipules entire 10

b. Stipules divided into 2-6 lobes 16

10*a.* Leaves 1.5 cm or shorter; ultimate divisions rhomboid 11

b. Leaves 3 cm long or shorter; ultimate divisions filiform to spatulate . . . 12

11*a.* Styles shorter than the ovary; leaves up to 8 mm long, 2 or 3 times forked; ultimate divisions 0.5-1.5 mm long . . . . 11. **P. rutifolium** Warming

b. Styles nearly as long as the ovary; leaves 5-10 mm long, once forked; ultimate divisions 2-4 mm long 12. **P. fruticulosum** (Tul. & Wedd.) Wedd.

12*a.* Ultimate divisions filiform 13

b. Ultimate divisions spatulate or linear 14

13*a.* Stipules entire, top irregularly shaped . . . 2. **P. ceratophyllum** Mx

b. Stipules divided into 2-6 narrow and acute lobes 4. **P. schenckii** Warming

14*a.* Fertile and sterile stems on different shoots along the same root; the fertile stem up to 3 mm high; sterile stem 20-60 cm high . . . . 3. **P. dimorphum** van Royen

b. Fertile and sterile stems on the same individual 15

15*a.* Ultimate divisions subfiliform to linear, 0.05-2 cm long; stipules with an irregularly formed top. . . . . 2. **P. ceratophyllum** Mx

b. Ultimate divisions spatulate, 0.5-1 cm long; stipules entire or shortly lobed; top or tops acute . . . . 5. **P. undulatum** van Royen

16*a.* Stipules wide, subamplexicaul, squamiform, coriaceous; leaves 1-7 mm long . . . . 6. **P. glaziovianum** Warming

b. Stipules narrow, concave, usually not amplexicaul, membranaceous . . . 17

17*a.* Ultimate divisions filiform or subfiliform 18

b. Ultimate divisions spatulate, quadrangular or linear 20

18*a.* Top of thecae acute; tepals about 1 mm long; stipules c. 1.5 mm long . . . . 7. **P. aguirense** Chod. & Visch.

b. Top of thecae obtuse; tepals 1-2 mm; stipules 0.5-5 mm long 19

19*a.* Inner loculi of the anthers shorter than the outer ones . . . . 2. **P. ceratophyllum** Mx

b. Inner loculi as long as the outer ones . . . 4. **P. schenckii** Warming

20*a.* Leaves 2-18 mm long, with rhombiform ultimate divisions . . . . 11. **P. rutifolium** Warming

b. Leaves 0.5-15 cm long, with spatulate to linear ultimate divisions 21

21*a.* Leaves 2-15 cm long; stems 0.1-80 cm high 23

b. Leaves 0.5-2.5 cm long; small herbs, stem not more than 1.5 cm high 22

22*a.* Stipules obtuse . . . . . . 10. **P. ricciiforme** (Liebmann) van Royen

b. Stipules acute . . . . 9. **P. ostenianum** Warming

23*a.* Fertile stems not more than 3 mm high, separate from the sterile ones but springing from the same root. Leaves of sterile plant 2-12 cm long, stems 20-60 cm high; ultimate divisions 2-6 mm long, nerveless. . . . . 3. **P. dimorphum** van Royen

b. Each specimen with flowers and leaves; stems 0.2-20 cm high. Leaves 1-20 cm long 24
24*a*. Ultimate divisions spathulate, abruptly widened at the top, one-nerved . . . . 5. **P. undulatum** van Royen
b. Ultimate divisions else and not abruptly widened at the top 25
25*a*. Top of thecae acute; tepals about 1 mm long; stipules 0.5-1 mm long . . . . 7. **P. aguirense** Chod. & Visch.
b. Top of thecae obtuse; tepals 1-2 mm long; stipules 0.5-5 mm long 26
26*a*. Inner loculi of the anthers shorter than the outer ones . . . . 2. **P. ceratophyllum** Mx
b. Inner loculi as long as the outer ones . . . 4. **P. schenckii** Warming
27*a*. Top of leaf non-apiculate 28
b. Top apiculate 29
28*a*. Stipules inserted near the top of the leaf-base, triangular to trapezoid, acute . . . . 16. **P. mülleri** Warming
b. Stipules inserted at the middle of the leaf-base, irregularly quadrangular to ovate, acute to obtuse . . . . 14. **P. uruguayense** Warming
29*a*. Leaf-base gradually passing into the petiole. Stems 1-10 cm high . . . . 15. **P. galvone** Warming
b. Leaf-base abruptly narrowed before passing into the petiole. Stems 2-10 mm high . . . . 13. **P. dentatum** van Royen

1. **Podostemum distichum** (v. Cham.) Wedd., (1873) 73–74; Warming (1888) 454–455, t. 19 f. 5–7; Warming (EP 1891) 21—*Podostemon distichus* (v. Cham.) Warming, Engler (1930) 63—*Lacis disticha* v. Cham., (1833) 653; idem, (1835) 504, t. 5—*Podostemon chamissonis* Tul., (1849) 103; idem, (1852) 133–135; Walpers (1852) 438; idem (1858) 784; Tul. (1863) 255–256, t. 74 f. 2—Plate 2 f. 7-13.

Small to medium-sized, 1–15 cm high herb, roots 3–4 mm wide. Stem compressed, flexuose, quadrangular, 1–2 mm diam, internodes 1–4 mm. Leaves repeatedly forked, 1–2 cm, petiole decurrent, triangular in transverse section, up to 5 mm long, stipule with 2 long, acute, inner teeth and 2 smaller, acute, outer ones, ultimate divisions of the leaf provided with 2–5, triangular, acute, sometimes 2–topped, up to 2.5 mm long irregularly whorled segments. Flowers not seen by me but according to Warming similar to those of the other species. Anthers emarginate with 2 unequal, emarginate thecae each containing two unequal loculi. Fruit obliquely ellipsoidal to globose, 1.5–2 × 1–1.5 mm, provided with 8 ribs, 2 of them very closely approaching each other, ribs on the caducous valve slightly S-shaped, on the persistent valve straight or slightly bent; pedicel 0.5–1 mm.
Type: Sellow s.n. in W.
Distr.: Brazil. (Prov. São Paulo, Sta Catharina and Rio Grande do Sul)

São Paulo — without loc., Sellow s.n., (BR, L, M, P, W); idem Gaudichaud 10 (P) — *Sta Catharina* — Rio Capivare, in Serra Geral, Ule 1877, Jan. (P) — *Rio Grande do Sul* — Serra dos Taypea, Schwacke 2675, March (C); Aparados da Serra (Bom Jesus), Fasenda Bernardo Velho, Rambo 34957, Jan. (S).

2. **Podostemum ceratophyllum** A. Michaux, (1803) 164, t. 44; Nuttall (1818) 202; Persoon (1819) 532; Sprengel (1825) 95; v. Chamisso (1835) 505; Hooker (1836) 23, t. 20; Schleiden (1839) 54, t. 7 f. 114–117; Torrey (1843) t. 98; Schnitzlein (1843) t. 85 f. 1–2; Tulasne (1849) 103; idem (1852) 129–132; Walpers (1852) 784;

idem (1858) 784; Weddell (1873) 72; Baillon (1888) f. 262; Warming (1881) t. 1–4; idem (1882) t. 7; idem (EP 1891) 21, f. 1; Chapman (1865) 39; idem (1897) 420–421; Nash (1905) 6; Engler (1930) 63, f. 1 & 18; Sprague (1933) 46; Hammond (1937) 417, 36 fgs; Moscoso (1943) 183.

var. **ceratophyllum**

Podostemum abrotanoides Nuttall (1834) 105-107—*P. ceratophyllum* Mx, var. *abrotanoides* (Nuttall) Wedd. (1873) 73—*P. ceratophyllum* Mx, var, *abrotanoides* (Nutt.) Fassett, (1939) 526—*P. ceratophyllum* Mx, var. *chondroides* Fassett (1939) 526—*P. ceratophyllum* Mx, var. *fluitans* Weddell (1873) 73—*Lacis ceratophylla* Bongard (1835) 78—*Aphyllon aquaticum virginiense petraeum* Plukenet (1692) t. 138, f. 1; idem (1696) 16.[1]

Small to large, unbranched or slightly branched, 4–20 cm high herb. Stem terete to subterete, flexuose, 0.5–1.5 mm wide. Leaves repeatedly forked, 1–20 cm, usually provided with a 0.5–5 mm long stipule, divided into 1–3, obtuse or acute, irregularly shaped lobes; ultimate divisions at first spatulate, obtuse, 0.5–1.5 mm, with a single indistinct nerve, afterwards filiform, subfiliform, linear, or spatulate and up to 20 mm long. Rests of leaves on the basal part squamiform, triangular, with a wide base, subamplexicaul, 0.5–1.5 mm, always provided with a stipule. Flowers few; pedicel 2–8 mm; juvenile spathella clavate, umbonate, mature one infundibuliform, up to 4 mm; tepals 2 or 3, filiform, acute, 1–1.5 mm, andropodium 1.5–2 mm, membranous, filaments 0.5–1 mm, anthers quadrangular, 1–1.5 mm, outer thecae longer than the inner ones, base of the thecae obtuse, connective widened and slightly longer than the thecae, sometimes bifid, pollen grains 2-celled, c. 40 μ long; ovary obliquely ellipsoid, 2–3 × c. 1.5 mm wide, elliptic in transverse section, provided with 8 ribs, styles 2, filiform, subpapillate, up to 1.5 mm. Fruit with one caducous valve, both valves with 5 nerves.
Type: Richard s. n. in P.
Distr.: NE Canada, S and E regions of the U.S.A., Honduras, Dominican republic.
Vernacular name: river weed (Northern America), ova (Dominica)

Canada: Ottawa-river, Allen s.n., Aug. (C, NY); Hull, Macoun s.n., (C, GH, MO, NY); Eel-river, near Woodstock, Macoun 22593, (F, GH); Brighams Creek, near Ottawa, Macoun 5907, (F, GH); St Eustache, M.-Victorin 3212, Aug. (GH, MO, NY); idem, Rivière des Mille-Iles, M.-Victorin 43791 (GH); Ile Bizard, M.-Victorin 22081, (F, GH, MO, P); Saint Joseph, Rivière-des-Prairies, M.-Victorin & Rolland Germain 33998, (C, F, GH); Rivière-des-Prairies, Brunel & Lanouette 45771, (GH).

United States: Maine — Kennebec Co., West Gardiner, Collin's Dam, Fassett 18295, fl. fr. Aug., (F, GH, MO, NY); Bradley, Chemostream, Merrill s.n., Oct. (NY); idem, Ricker 628, Oct. (US); Penobscot Co, Gilman falls, Ogden & Babel 2178, Aug. (GH); idem, Steinmetz & Ogden 840, fl. fr. Aug. (F, GH, MO, NY, P, S, US) — Vermont — West river, Jamaica, Dobbins s.n., fl. fr. Aug. (GH); Woodstock, Winslow s.n., July (US) (Massachusetts — Stony brook, Blankinship s.n.,

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[1] The literature has been restricted to the more important items as this species is incorporated in practically every handbook dealing with the North American flora.

(GH); Southampton, Chapman s.n., (MO); South Natick, Charles river, Faxon s.n., fl. fr. July (C, GH, US); idem, E. & C. E. Faxon s.n., Aug. (GH, NY); South Natick, Morong s.n., Sept. (NY, US) — Rhode Island — East Providence, Hunts Mill, Collins s.n., (GH, MO, US); idem, Leland s.n., Aug. (MO); idem, Ten Mile river, Williams s.n., Aug. (GH); Cumberland, Olney s.n., July (GH, NY) — Connecticut — Pequonnock river, Eames 11722, fl. fr. Aug. (GH); Mill river at Samp Mortar, Johnson s.n., fl. fr. April — New York — Jefferson Co, Cramer s.n., fl. fr. (GH); Watertown, Cramer 1 A, (GH); Franklin Co, St Regis river, Muenscher & Maguire 1197, fl. fr. Sept., (F, GH, MO, S); idem, Chateaugay Chasin, Muenscher & Maguire 1196, Sept., (GH); idem, Muenscher & Justice 839, fl. fr. Aug. (F, GH, MO, NY, P, S); St Lawrence Co., Grasse river below Massena, Muenscher & Maguire 1198, Sept. (F, GH, MO, US); Oneido lake, Sartwell s.n., (F); idem, Wright s.n. (F) — Pennsylvania — Allegheny Creek, Berks Co., Brumbach 670-33, (GH); Water Gap, Britton s.n., (BR, C); Brandywine near Westchester, Darlington s.n., (GH); Pike Co., Winona falls near Bushkill, Fassett 19486, Sept. (F, GH); idem, Fassett & Calvert 19488, Sept. (F, GH, MO, P); Delaware river, near Easton, Garter s.n., (NY); idem, Porter s.n., Aug. (F, GH); Cuihuita river, Gattinger 2468, Aug. (F, MO, NY); Delaware Co., Chester Creek, Pennell & Adams 591, Sept. (GH, MO); Chester Co., Canby s.n., fl. fr. (F, P); Monroe Co., Marshall Creek, Howenke 19, Aug. (NY); Northampton Co., Raw s.n., (MO); idem, Martius creek, Williamson s.n., Aug. (NY); without loc., Gray s.n., fl. fr. (P, S); idem, Lesquereux 491, (C); idem, Schweinitz s.n., (BR, P) — Ohio — Ohio river near Louisville, Richard s.n., fl. fr. (B, P) — New Jersey — without loc., Austin s.n., (NY); — Delaware — Brandywine creek, Wilmongton, Canby s.n., fl. fr. (GH); idem, Commons s.n., Aug. (US); Red Clay creek, Greenbank, Commons s.n., June/Sept., (MO, NY, US) — Maryland — Swallow falls of Deep Creek, Garrett Co., Drouet 2346, Aug., (F); Prince George Co., Patuxent river, Hermann 9940, Oct., (F, NY); idem, Anocosta river, Hotchkiss 7107, July (US) — District of Columbia — Potomac river, Holm s.n., June (US); Chain bridge, Holm s.n., June (S); Howard Co., Patuxent river, House 1521, Sept. (MO) — Virginia — Bull Run below Beverley Hill, Allard 8152, (GH); Guyandott river, Berkley 431, (MO); Cacapou river near Wardensville, Berkley 1574, (MO); Catawba river, Burke s.n., (NY); Bedford Co., Curtiss s.n., fl. fr. July (F, MO, S); idem, Curtiss 7668, fl. fr. July (GH, MO); Twelve Pole Creek, Plymale 620, Aug. (GH) — Kentucky — Whitley Co., Cumberland, Cumberland falls, Braun 2623, (GH, NY); idem, Clarke s.n., (US); idem, Mac Farland 95, fr. Oct., (GH, MO, NY); Harlan Co., Harlan Court House, Kearney 19, Aug., (GH, NY, US); Kentucky river, Peter s.n., July (L); Lexington, Short s.n., (NY) — North Carolina — Frenchbroadriver, Hot Springs, Biltmore 738, (NY); idem, Beyrich s.n., (C, L, MO, P); Person Co., Earl Hall s.n., Febr. (NY); Tuckazeigee falls, Thaxter s.n., (GH); Rutherford Co., Rainbow falls, Wherry s.n., March (NY); Asheville, Beyrich s.n., (MO); without loc., Schweinitz s.n., (BR, P) — South Carolina — Chutu Co., Landsford, Catawba river, Green 3, (F) — Tennessee — Tellico Plains, Tellico river, Everman s.n., Oct. (F); Unana Mts, Cattinger s.n., July (F, GH, NY, US); Conaranga river, Gattinger s.n., Sept. (US); idem, Gattinger s.n., Aug. (GH); Pigeon river, Sevierville, Svenson 4007, Aug. (GH); Grundy Co., Collins river below Altamont, Aug. (GH); Little river, Gattinger s.n., July/Aug., (MO, US); Green Co., Paint Rock creek, Redfield 7669, Aug. (L, MO) — Arkansas — Montgomery Co., Caddo Gap, Watts & Fassett 18693, April (F, GH, MO, NY) — Mississippi — Chickasawha river, Enterprise, Tracey 3257 & 3258, Dec. (NY); Meridion, Tracey 3259, Febr. (US); idem, Tracey 3262, March (NY) — Alabama — Covington Co., Patsaliga creek, near River Halls, Haroer 106, June (GH, MO, NY); Mussel Shoals, Lauderdale Co., Tennessee river, Harper s.n., Oct. (US); idem, near Florence, Harper s.n., Oct. (NY); Butler creek, Lauderdale Co., Harper 3870, April (F, GH, MO, US); Goosa river, Chiokwa creek, Rugel s.n., Sept. (C, MO, NY) — Georgia — Tallotah falls, Ascherson 19 (P); Chattahoochee river, Boykins s.n. (NY); idem, near Columbus, Nuttall s.n. (NY); Flint river, near Albany, Chapman s.n. (NY); Goosa river, Chapman (F, GH, MO); Dougherty Co., Muckafoonee creek, Harper 1950, fl. fr. Aug. (F, GH, MO, NY); Rabun Co., Tallulah falls, Seymour s.n., Sept. (GH); idem, Small s.n., Sept. (F, MO, NY); Estotoak falls, Small s.n., Aug. (NY); without loc. Torrey s.n., fl. (GH, MO, NY, P); Warm Springs, Beyrich s.n., (MO); Oglethorpe

Co., S. Broad river, Pyron & Mac Vaugh 41, Nov. (GH, MO, US) — Wyoming - Wyoming Co., Guyandot river, below Baileysville, Morris 1210, (F, US) — State unknown — Little river, Oxford Co., Harper s.n., Aug. (GH); Onigham (?) creek, Hull, Scott s.n., Aug. (C); Delto creek, Garrett Co., J. D. Smith s.n., July (US) — Without loc. — Léman s.n., fl. fr. (P); Joor, (MO); Thurber s.n. (GH).

Dominican Republic: Prov. Santiago — distr. San José de las Matas, Rio Magua, near Moncion (Guaraguano), Valeur 130 (C, F, MO, S and herb. San Domingo); idem, Ekman H 12640, May (S); idem, Ekman H 14600, April (S).

var. **circumvallatum** v. Royen, nov. var.—P. 258

Differs from var. ceratophyllum in the shorter rigid and coarse stems, the densely congested and more numerous rests of leaves on the basal part of the latter and the obtuse stipule. It grows moreover in a different region. Flower and fruit are unknown.
Type: Standley 56080 in US.
Distr.: Honduras. (Department Comayagua)

Siguatepeque, Rodriguez 2671, April (F); idem, Standley & Chacon 6158, 6451 & 6463, March/April (F); idem, Standley 56071 & 56080, Febr. (F).

3. **Podostemum dimorphum** v. Royen, nov. sp.—P. 258 and plate 2 f. 14–16.

Sterile and fertile individuals on different stems springing from the same root. Sterile individuals 20–60 cm high, stem 2–3 mm diam, unbranched, lax, sometimes slightly winged, compressed. Leaves bipinnate, 2–12 cm, main axis terete or subterete, 1–1.5 mm diam, pinnae of the first order pinnate or forked, ultimate divisions linear, 2–6 mm, stipule inserted with a broad base, concave, membranous, 2–3 mm long, 1–1.5 mm high, with 1 or 2 acute tops. Fertile individuals stemless or with an up to 3 mm high stem, leafless, with markedly widened, lobed bases, subopposite along 2–3 mm wide, compressed roots, each individual provided with a single flower. Flower known in bud only; spathella clavate, umbonate, 2–2.5 mm, rigid, somewhat coarse; tepals 3, linear to filiform, acute, two of them c. 1 mm, the third one in the fork between the two filaments and slightly shorter than the other two, andropodium quadrangular, membranous, c. 1 × 0.5 mm, filaments c. 0.8 mm, membranous, widened at the top, anthers irregularly quadrangular, 1 mm long, top widely emarginate, base emarginate, thecae unequal, obtuse, pollen grains 28 × 15.6 μ, 2-celled; ovary globose, 0.5 mm diam, incompletely known, styles filiform, acute, 0.5 mm. Fruit unknown.
Type: Dusén 16540 in C.
Distr.: Brazil.

Moringava, near Itarapé, Dusén 16540, fl. buds Jan., (C, S, U).

4. **Podostemum schenckii** Warming, (1888) 451–454, 480, t. 18 f. 1–18, t. 19 f. 1–4; idem, (EP 1891) 21; idem (1899) 128–129, f. 24; Engler (1930) 63; Herter (1930) 65—Plate 2 f. 17–19.

Small to medium-sized coenobia, the individuals subopposite along 1–2 mm wide, compressed, strongly branched roots. Stems 0.5–15 cm high, 1–2.5 mm diam., quadrangular, winged, slightly branched in the basal parts, more strongly branched towards the top, internodes 0–20 mm. Leaves a few times forked, 0.8–11 cm; petiole narrow, 0.4–4 cm, terete to quadrangular but triangular and sulcate at the base, subamplexicaul and decurrent, margins membranous, ultimate divisions of the leaf spatulate to linear, filiform or subfiliform, nerveless, 2–10 mm, provided with a 1–2.5 mm long stipule which is inserted with a broad base and divided into 2–6, from 0.5 to 1.5 mm long acute lobes, concave, subamplexicaul, basal stipules often subobtuse and 2-lobed, sometimes shifted to the margin. Basal rests of leaves squamiform, convave, with obtuse, irregularly shaped top and amplexicaul base, the lowest ones with an indistinct, narrowly concave, acute or obtuse stipule. Flowers terminal or axillary, solitary; pedicel 2–3 mm; juvenile spathella clavate, umbonate, membranous, mature one campanuliform to infundibuliform, 2–3 mm; tepals 3, filiform, two of these 1.5–2 mm, the 3rd tepal 1–1.5 mm, andropodium membranous, keeled, with a single distinct nerve, 1–3.5 mm, filaments 0.5–1.5 mm, anthers 1–1.5 mm, top and base emarginate, thecae unequal, top of thecae obtuse, base acute, pollen grains 2-celled, 32.6 × 22.5 μ; ovary ellipsoid, 1.5–2 × c. 1 mm, consisting of 2 oblique, unequal cells and provided with 8 ribs, but the sutural ribs very closely approaching each other, ribs straight or slightly bent, styles 2, pyramidal, with a narrow base, c. 0.5 mm, acute. Fruit with 2 valves, each valve with 5 ribs.

Type: Schenck 328 in C.

Distr.: Brazil. (Province Goyaz, Minas Geraes, Sao Paulo, Santha Catharina, Rio Grande do Sul) and Uruguay.

Brazil: Goyaz — Rio Trinidade, Glaziou 21994, fl. fr. Aug. (BR); Rio Douadinha, between Panga and João Visva, fr. Aug. (BR, C, F, P, S, US) — Minas Geraes — Rio Bugres, Mosén 738, Oct. (S) — Sao Paulo — Rio Piracicaba, Glaziou 19818, (C, P) — Santha Catharina — Salto, near Blumenau, Schenck 328, fl. fr. Sept. (C); Rio Itayahy, near Blumenau, Schenck 580, Oct. (C); Rio Tubarão, near Pedras Grandes, Ule 1875, July (P); Rio Capinzal, near Santha Catharina, Dusén 17870, fl. fr. Febr. (C, S); without loc., Schenck s.n., (C) — Rio Grande do Sul — Serra dos Tapes, cascade de Hermenogilda, Lindman A 775, Dec. (C, S); Silveira Martius, Lindman 1295, March (C, S) — Without loc. — Schwacke 6069, (C); Schwacke 2874, (C); Glaziou A & B, (C).

Uruguay: Rio Negro, Berro 3, March (C); Salto Grande, Berro 3328, April, (C); Barra de Vera, Berro 3394, 5158 & 5161, Febr. (C).

5. **Podostemum undulatum**, nov. sp., var. **undulatum**—P. 259 and plate 2 f. 20.

Small, slightly branched individuals, 2–5 cm high, opposite along thin, c. 1 mm wide roots; internodes subcompressed, 1–3 mm, flexuose, fragile. Leaves a few times forked, 2–7 cm, petiole 0.1–3 cm long, 0.5–1 mm in diam, subterete, triangular at the base, slightly decurrent, ultimate divisions spatulate, 5–10 × 0.2–0.5 mm, abruptly widened at the top, subacuminate, with a single distinct nerve, membranous, margins undulate, stipules concave, 1–2 mm; subamplexicaul; apical stipules undivided, basal ones with 2 lobes which are 0.5–0.8 mm long, andropodium c. 0.5 mm membranous; filaments 0.5 mm, widened

at the top, anthers with unequal thecae, c. 1 mm long, top obtuse or emarginate, base of thecae obtuse; pollen grains 2-celled, imcompletely known; ovary ellipsoid, c. 0.5 × 0.5 mm, provided with 8 ribs, of which 2 approach each other very closely; styles filiform (?), cohering at the base. Fruit unknown.
Type: Ule 804 in P.
Distr.: Brazil, Uruguay and Paraguay.

Brazil: Rio Itayahy, near Blumenau, prov. S. Catharina, Ule 804, fl. April; Rio Itapua, prov. Corrientes, Bonpland s.n., (P).
Uruguay: Salto Chico, Uruguay river, dept. Salto, Herter 1708, Nov. (F, GH, MO, U, US).
Paraguay: between Rio Apa and Rio Aquidaban, Fiebrig 4990, (GH, M).

var. **angustifolium**—P. 259

Differs from the variety *undulatum* in its dimensions, i.e. in the 3–12 cm high stem, in the 5–8 cm long leaves, in the narrower, spatulate, ultimate divisions and in the longer, 1–3 mm long, stipules.
Type: Jörgensen 4989 in C.
Distr.: Known from the type-locality only.

Mbuvera, Arroyo Iguazu, Jörgensen 4989, May (C, F, MO, NY, S).

6. **Podostemum glaziovianum** Warming, (1899) 130–133, f. 26–28; Engler (1930) 63—Plate 2 f. 21–25.

Small, slightly branched individuals, subopposite along thin roots, prostrate or ascending. Stem up to 4 cm long and up to 2 mm diam, twisted, compressed. Leaves far apart, but the bases in the flowering region overlapping, a few times forked, up to 7 mm, ultimate divisions subfiliform, 0.5 mm long or less, petiole up to 2 mm, widened at the base, subamplexicaul, stipule widely squamiform, with 2 acute or obtuse tops, up to 1 × 1.5 mm[1]. Basal rests of leaves squamiform, with a similar stipule as in the complete leaves. Juvenile spathella clavate, umbonate, firm, mature one campanuliform, up to 1.5 mm, pedicel up to 1.5 mm, tepals 2 or 3, lanceolate, two of them 0.8–1.5 mm, the third one sometimes absent, shorter than the other two, andropodium membranous, rectangular to cuneate, c. 1 mm, anthers obliquely quadrangular, c. 1 mm, top and base emarginate, outer thecae longer than the inner ones, pollen grains 2-celled, 26.6 × 15.9 μ; ovary ellipsoid, c. 1.5 × 1 mm, provided with 8 ribs, styles filiform, 0.5 mm. Fruit slightly exceeding the spathella; with one valve caducous.
Type: Glaziou 21993 in C.
Distr.: Known from the type-locality only.

Rio Trinidade and Rio Cassu, Glaziou 21993, fl. fr. Aug. (C, P).

7. **Podostemum aguirense** Chodat & Vischer, (1917) 240–241, f. 195–196—*Podostemum warmingii* Chodat & Vischer, l.c. 240, f. 176–180, 183, 188–192, 194—Plate 3 f. 1–6.

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[1] The leaves are said (WARMING) to bear numerous fine threads, but in the type-specimens these are not to be found. WARMING's threads may have been minute algae such as were found by WENT on some Suriname Podostemaceae.

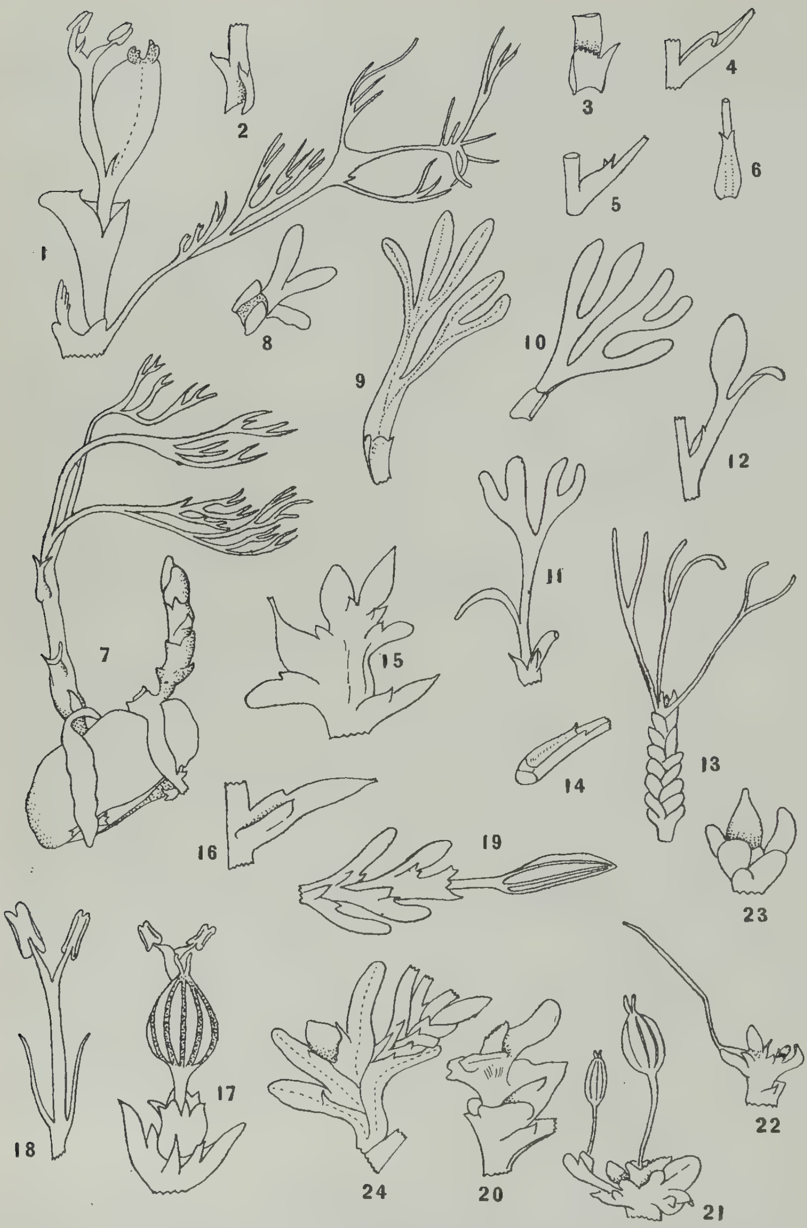

PLATE 3 — 1-6. **Podostemum aguirense** Chod. & Visch. *(Chodat & Vischer 338)*, 1. apical part of shoot; 2-3. stipule; 4-6. stipules, different views, (1-3 according to a drawing made by Chodat and Vischer) — 7. **Podostemum atrichum** Chod. & Visch. *(Chodat & Vischer 337)*, roots with shoots (made after a drawing by Chodat and Vischer) — 8-9. **Podostemum ostenianum** Warming *(Osten 2903)*, leaves with stipules — 10. **Podostemum ricciiforme** (Liebmann) v. Royen

Coenobia consisting of small shoots developing along 1–2 mm wide, flattened roots. Stem 0.1–7 cm, terete or quadrangular, c. 2 mm diam, branched or unbranched, with a few leaf-bases at the base and some complete leaves at the top. Basal rests of leaves squamiform, deeply and narrowly concave, amplexicaul, 1.5–2.5 mm, at either margin with a single short, obtuse, sometimes indistinctly developed tooth, on the outer surface with hard papillae, sometimes in the lower part of the stem united with each other. Leaves a few times forked, 1–3 cm, at the base with a c. 1.5 mm long stipule, which ends in 2 acute, 0.5–1 mm long teeth, petiole 2.5–10 mm long, c. 1 mm wide, compressed, concave at the base, ultimate divisions of the leaf very narrow, spatulate, acute, 2–6 mm. Flowers few, pedicel 2–5 mm, juvenile spathella clavate, umbonate, papillate, mature one widely campanuliform; tepals 3, linear, acute, two of them c. 1 mm, the third tepal sometimes absent, andropodium membranous, provided with a single nerve, sometimes subcarinate, 1–2 × c. 0.5 mm, filaments up to 0.5 mm; anthers c. 1 mm, quadrangular, top truncate, emarginate, base incised to one fourth, thecae unequal, acute at the top, base emarginate, pollen grains 2-celled, 24.9 × 15.9 μ; ovary ellipsoid, c. 2 × 1 mm, terete, indistinctly 8-ribbed, styles at first pyramidal, afterwards subulate, compressed, acute, papillate, up to 1 mm. Fruit 2–3 mm, each valve provided with 5 straight, prominulous ribs.
Type: Chodat & Vischer 338, in G–Del.
Distr.: Argentina, Brazil and Uruguay.

Paraguay: Salto Grande Yguazu, Chodat & Vischer 338 & 339, fl. fr. Oct. (G-Del).
Brazil: Prov. Minas Geraes, Ribeirao da Caquiero, Regnell III 2053, April (P).
Argentina: Prov. Misiones, Yguazu, Osten & Rojas 8165 & 9166, Sept. (S).

8. **Podostemum atrichum** Chodat & Vischer, (1917) 241–242, f. 169–172, 181–182, 187—Plate 3 f. 7.

Small individuals arising from 1–1.5 mm wide, flattened roots. Stems opposite or subopposite, 0.1–7 cm high, quadrangular, compressed, 4-ribbed, branched or unbranched, at the base with the rests of leaves provided with a 1- or 2-topped intrapetiolar stipule. Leaves a few times forked, 1–3 cm, provided with a narrowly concave, amplexicaul base; stipule ending in three 1–1.5 mm long, acute teeth; petiole c. 1 cm long, compressed, sulcate at either side; ultimate

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(Liebmann s.n.), leaf with stipules — 11-12. **Podostemum rutifolium** Warming *(Schwacke 5053)*, leaves with stipules — 13-14. **Podostemum fruticulosum** (Tul. & Wedd.) Wedd. *(Weddell s.n.)*, 13. habit; 14. part of petiole, with stipule — 15-19. **Podostemum dentatum** v. Royen *(Ule 1876)*, 15. apical part of shoot; 16. basal part of leaf with stipule; 17. apical part of a shoot with flower; 18. androecium; 19. apical part of shoot with part of fruit — 20-23. **Podostemum uruguayense** Warming *(Osten 2904)*, 20. part of shoot; 21. apical part with flowers; 22. idem, with complete and incomplete leaves; 23. idem, with closed spathella — 24. **Podostemum mülleri** Warming *(Müller s.n.)*, apical part of shoot with closed spathella.

divisions lanceolate, acute, nerveless, sometimes with an indistinct nerve, 1–9 × c. 0.2 mm. Flowers and fruit unknown.
Type: Chodat & Vischer 337 in G-Del.
Distr.: Paraguay and Brazil.

Paraguay: Salto Grande Yguazu, Oct. (G-Del.).
Brazil: Prov. Rio Grande do Sul, Arroio Alegre, Bornmüller 738, Oct. (GH).

9. **Podostemum ostenianum** Warming, (1899) 127, f. 23; Engler (1930) 63; Herter (1930) 65—Plate 3 f. 8–9.

Small, weak, up to 2 cm high individuals, opposite or subopposite along compressed, up to 2 mm wide, repeatedly branched roots. Stems up to 1.5 cm, subcompressed. Leaves a few times forked, 0.4–2.5 cm, petiole sheath-like at the base, amplexicaul, stipules either 2, one on either side of the petiole, and then triangular and acute, or one only and then concave and divided into two lobes, ultimate divisions of the leaf spatulate, acuminate, with a single distinct nerve or nerveless, c. 1 × 0.5 mm. Flowers terminal, pedicel up to 5 mm; juvenile spathella clavate, umbonate, coriaceous, mature one tubuliform, up to 3 mm, tepals 3, filiform, two of them 0.5–1 mm, the third tepal in young stages larger than the other two, andropodium membranous, 2–2.5 mm, filaments subulate, anthers irregularly quadrangular, 0.5–1 mm, top of thecae obtuse, emarginate, base acute, pollen grains 2-celled, incompletely known; ovary ellipsoid, 2.5 mm high or shorter, subacute, styles cylindrical, acute, up to 1 mm. Fruit similar to the ovary, each valve with 5 ribs.
Type: Osten 2903 in C.
Distr.: Uruguay and Paraguay.

Uruguay: dept. Salto, Salto Grande, Uruguay river, Osten 2903, fl. fr. Dec. (B, C); idem, Berro 5160 & 5168, March (C); Barra de Vera, Berro 5157, Febr. (C).
Paraguay: between Rio Apa and Rio·Aquidaban, near Caballero-ricé, Fiebrig 4990, Febr. (L).

10. **Podostemum ricciiforme** (Liebmann) v. Royen, nov. comb.—*Marathrum ? ricciaeforme* Liebmann, (1849) 512; Warming (1901) 49—Plate 3 f. 10.

Similar to P. ostenianum, but stipule hardly visible and provided with 1 or 2 obtuse tips. Flowers and fruits unknown.
Type: Liebmann s.n. in C.
Distr.: Mexico and Costa Rica.

Mexico: Baranca de Huitamalco, dept. Vera Cruz, Liebmann s.n., May (C).
Costa Rica: without loc., Endris (?) 181, (W).

11. **Podostemum rutifolium** Warming, (1899) 129–130, f. 25; Engler (1930) 63; Herter (1930) 65—Plate 3 f. 11–12.

Small, up to 7 cm high individuals with branched stems, opposite or subopposite along 2–3 mm wide compressed roots. Stems compressed, 1 mm diam. Leaves a few times forked, up to 8 mm; petiole

3–5 mm, amplexicaul, stipule concave, c. 0.5 mm, undivided or sometimes with two acute tips, ultimate divisions of the leaf ovate to quadrangular, obtuse to subacute, 0.5–1.5 mm, nerveless. Flowers terminal, pedicel 3–5 mm, juvenile spathella clavate, umbonate to acute, coriaceous, mature one infundibuliform, 3–4 mm, tepals 3, linear, acute, two of them 1–1.5 mm, but the third tepal c. 1 mm; andropodium membranous, 1 × 0.5 mm, filaments 0.8 mm, anthers obliquely quadrangular, obtuse, base emarginate, outer thecae larger than the inner ones, in either case top obtuse, base acute, pollen grains 2-celled, yellowish, 34.7 × 21.3 μ; ovary ellipsoid, obtuse, up to 2 mm high, provided with 8 lighter coloured ribs, styles 2, pyramidal, base broad, top acute, 0.5 mm long or less. Fruit unknown.
Type: Schwacke 5053 in C.
Distr.: Brazil and Uruguay.

Brazil: Rio Itayahi, near Blumenau, Schwacke 5053, fl. (C); Garcia brook near Blumenau, Schenck 187, Sept. (C).
Uruguay: Rio Negro, Berro 2, March, (C); Salto Grande, Uruguay river, Berro 5199, April (C).

12. **Podostemum fruticulosum** (Tul. & Wedd.) Wedd., (1873) 74; Engler (1930) 63—*Castelnavia fruticulosus* Tul. & Wedd., Tul. (1849) 110; Tul. (1852) 176–178, t. 12 f. 3; Walpers (1852) 442; idem (1858) 790; Tul. (1863) 270–272, t. 76 f. 3—Plate 3 f. 13–14

Small, erect individuals with shoots in groups along thin roots. Stem 5–15 × 0.5–1 mm, unbranched. Leaves forked, 5–10 mm, petiole subterete, amplexicaul, stipules 2, inconspicous, marginal, obtuse, ultimate divisions 2, subfiliform to linear, 2–4 mm, obtuse. Rests of the leaves at the base of the shoot squamiform, close together, with the base enveloping each other, without stipule. Flower known in bud only, terminal, juvenile spathella clavate, membranous, stamens free or borne by an andropodium, tepals 3, two of them half as long as the stamens or the andropodium, the third one shorter; ovary ellipsoid; styles filiform, nearly as long as the ovary, markedly exceeding the spathella. (The description of the flower is extracted from the publication of WEDDELL as I was unable to find flowers).
Type: Weddell s.n. in P.
Distr.: Brazil, prov. Rio de Janeiro, Rio Piabanha in Serra da Estrella.

13. **Podostemum dentatum** v. Royen, nov. sp.—P. 259 and plate 3 f. 15–19.

Small, slightly branched or unbranched individuals, subopposite or opposite along thin roots. Stems 2–10 mm. Leaves entire, 0.5–2.5 cm × 0.2 mm, entire (or bifid?), base 0.5–2 × 0.5–1 mm, obliquely inserted, abruptly narrowed into the linear limb, which is provided with a single nerve, stipule triangular to ovate, acute, 1–1.5 × 0.5 mm, inserted with a narrow, linear base in the middle of the base of the leaf, top of the base of the leaf in the apical leaves provided with a short,

triangular, 0.5 mm long tooth. The squamiform rests of the leaves at the base spatulate, 0.5–2 × 0.5–1 mm. Flowers few, terminal and axillary, pedicel 1–3 mm, juvenile spathella clavate, umbonate, coriaceous, mature one campanuliform, 2–3 mm, tepals 2, the third one unknown, linear, acute, 0.5–1.5 mm, andropodium membranous, 2–2.5 mm, filaments lanceolate, 0.5 mm long, anthers sagittate c. 1 mm, with deeply emarginate top and base, thecae unequal with acute tops and subacute to acute bases, pollen grains 2-celled, 31.7 × 17.3 μ; ovary ellipsoid to ovoid, 2–2.5 × 1–1.5 mm, with 8 indistinct, wide, flattened ribs, styles filiform, 0.5–0.8 mm. Fruit with one persistent valve provided with 5 straight or slightly bent ribs, and one caducous valve with 5 slightly sinuous ribs.
Type: Ule 1876 in P.
Distr.: Brazil. (Prov. Santha Catharina)

Rio Tubarao, on Pedras Grandes, Ule 1876, fl. fr. July (P).

14. **Podostemum uruguayense** Warming, (1899) 133–135, f. 29 & 30; Engler (1930) 63; Herter (1930) 65—Plate 3 f. 20–23.

Small, up to 4 cm high individuals, subopposite along compressed roots. Stem twisted, compressed. Rests of leaves 4–8 × 2–5 mm, widely ovate to elliptic, obliquely inserted, amplexicaul, decurrent, sometimes keeled at the upper surface, rigid, abruptly passing into a narrowed, entire or a few times forked, up to 2 cm long limb with spatulate, ultimate divisions, stipule irregularly quadrangular to ovate, acute to obtuse, inserted with a narrow, laterally flattened base near the top, in the lower leaves shifted towards the middle of the leaf-base. Flowers few, along brachyblasts or axillary, pedicel 3 mm, juvenile spathella clavate, umbonate, mature one infundibuliform, 3–4 mm, coriaceous, not or very slightly exceeding the leaf-base, tepals 3, linear, acute, two of them 1–2 mm, but the third one at the most but slightly more than 1 mm, andropodium membranous, 1–3 mm, its midrib winged and decurrent along the pedicel, filaments subulate, compressed, 0.2–1 mm, anthers sagittate, c. 0.5 mm, top obtuse to subacuminate, base deeply emarginate, pollen grains 2-celled, 32.9 × 17.1 μ; ovary ellipsoid, 2–2.5 × c. 1.5 mm, provided with 8 flat, prominent ribs, styles conical, papillate, up to 0.5 mm. Fruit similar to the ovary, both valves with 5 ribs.
Type: Osten 2904 in C.
Distr.: Uruguay and Brazil.

Uruguay: dept. Salto, Salto Grande, Uruguay river, Osten 2904, fl. fr. Dec. (C); idem, Felippone 5255, fl. fr. (K, U).

Brazil: prov. Santha Catharina, Rio Itayahy, near Blumenau, Schwacke 5013, (C).

15. **Podostemum galvone** Warming, (1888) 450–451, t. 17 f. 16–20; Engler (1930) 63—Plate 4 f. 1–6.

Small, strongly branched individuals, subopposite or opposite along c. 2 mm wide roots. Stem 1–10 cm, subcompressed, fertile specimens shorter than sterile ones. Leaves entire or once or twice forked, up to

11 mm, sulcate at the base, subamplexicaul, decurrent, ultimate divisions spatulate, obtuse, up to 3 mm, margin undulate, with a single distinct nerve, stipules either two and then one at either margin of the base of the leaf, or but a single one, which is triangular, acute, c. 0.5 mm, base of the leaf gradually passing into the wider limb. Flowers few, pedicel 1–2 mm; juvenile spathella clavate, umbonate, coriaceous, mature one infundibuliform, 2–3 mm, tepals 3, linear, acute, two of them 1.5–2 mm, but the third one only c. 1 mm, anthers sagittate, 0.5–0.8 mm, acute, pollen grains 2-celled, 26.1 × 16 μ; ovary ellipsoid, 1.5–2 × 1 mm, with subtruncate top, and 8 indistinct ribs, styles subpapillate, acute, 0.5 mm long. Fruit similar to the ovary.
Type: Puiggari 852 in C.
Distr.: Brazil and Paraguay.

Brazil: Rio Iguape, Iporanga, Puiggari 852, fl. fr. July (C); idem, Puiggari 889, (C); idem, Puiggari 279, (P); idem, Rocken 415 (?), Sept. (C); near Apiahy, Matas-Altas, Rocken 272, March (C); without loc., Glaziou 16358, (P); idem, Glaziou 16359, (C, F, P).
Uruguay: Uruguay river, Salto Grande, Berro 5159, April (C); idem, Salto Chico, Berro 5170, April (C).

16. **Podostemum mülleri** Warming, (1899) 445–449, 480, t. 16 f. 1–9, t. 17 f. 1–15; Engler (1930) 63, f. 7—Plate 3 f. 24.

Small, branched, up to 7 cm high individuals. Stem twisted, compressed, quadrangular. Leaves with a wide base which abruptly narrows into the up to 8 cm long, entire or repeatedly forked limb; base ovate to elliptic, obliquely inserted, decurrent, coarse, 2–5 × 0.5–2 mm, stipule near the top or slightly shifted towards the middle, triangular to trapezoid, acute, 0.3–1 mm, inserted with a narrow, laterally flattened base, ultimate divisions filiform. Flowers numerous, pedicel 2–4 mm, juvenile spathella clavate, umbonate, coriaceous, mature one infundibuliform, up to 4 mm, tepals 3, linear, acute, two of them 1.5–2 mm, but the third one shorter and narrower, andropodium membranous, c. 1 mm, filaments subulate, 1–2 mm, anthers obliquely quadrangular, 0.5–1 mm, top truncate, emarginate, pollen grains 2-celled, 30.8 × 16.3 μ; ovary ellipsoid, up to 2 mm high, provided with 8 ribs, styles pyramidal to filiform, 1 mm, subpapillate. Fruit similar to the ovary, each valve with 5 ribs.
Type: Müller s.n. in B.
Distr.: Brazil (Provinces Santha Catharina, Sao Paulo, Rio Grande do Sul)

Santha Catharina: Rio Itayahy, Müller s.n., fl. fr. (B); idem, Schwacke 5055, (C); idem, near Blumenau, Schwacke 5010, (C); idem, idem, Ule 862, Aug. (P, US); idem, Schenck 330, 331 & 332, Sept. (C); Rio Garcia, near Blumenau, Schwacke 5012, (C); idem, Jordans brook between Garcia and Blumenau, Schenck 186, Sept. (C) — *São Paulo:* Rio Piracicaba, Glaziou 19816, fl. fr. (C, P); Rio Iguapé, Iporanga, Puiggari 86, July (C); idem, Puiggari 279, July (P); without loc., Glaziou 15443, 15444 C, 16358, (C, P); idem, Glaziou 16359, (P) — *Rio Grande do Sul:* Arroio Alegre, Bornmüller 737, fl. Oct. (GH) — *Without loc.:* Glaziou s.n., (P); Løfgren s.n., (C).

17. **Podostemum comatum** Hicken, (1917) 150–151.

As I had not seen the material I was unable to include this species in the key and to provide a description. For this reason I reproduce here, with a few corrections, the Latin description given by Hicken:

Caules duplicis naturae; alii steriles, repentes, herbacei, laeves, compressi, 2.3–4 mm lata et 20–30 cm longi flexuosi e margine singulo tantum folia multisecta petiolata fere ad nervos ramosos reducta emittentes. Caules alii fertiles, erecti, herbacei sed magis rigidi et rugosi, minus compressi, 6–8 cm alti, simplices, sursum foliiferi et floridi, deorsum nudi, sed stipulis auriculiformibus vel vaginiformibus caulem semi-amplectentibus. Lamina multifida, ad lacinias filiformes ramosas reducta usque ad 10 cm longa. Folia ramorum fertilium similia sed multo minora, petiolo brevissimo 2–3 mm longo et laciniis 20 mm longis, alternis et distichis, stipulis 2 semi-amplexicaulibus brevibus. Flores solitarii axillares in foveola caulis insiti. Spathella duriuscula cum clausa pyriformi, obovata. Androceum pedicelli apice laterali inseritur; filamenta 3 libera, lateralia castrata filiformia subulata, fertili multo breviora ovarium medium attinguentia. Filamentum bifurcum parte communi ovario duplo longius et intra crura appendicem subulatum, filiformem, brevem gerens; brachiis 1.5 mm longis, antheriferis. Antherae ellipsoideum sessile biloculare stigmatibus 2 linearibus planis liberis ornatum. Capsula matura pedicellata 1.25 mm longa, 8-costata; semina numerosa badia compressa.

Type: Rodriguez 791, Rio Iguazu, Argentine. fl. fr. April.

DUBIOUS SPECIES

1. **Podostemum** ? **dichotomum** Klotsch, Schomburgk (1848) 930.

This species is reported from the Essequibo, Mazaruni and Cuyuni river but as no specimens are indicated I was unable to trace this species.

6. CASTELNAVIA Tul. & Wedd.

Very small to small, liverwort-like herbs. Leaves known in a few species only, and these rather variable. Flowers numerous, in cavities of the thalloid base, distinctly zygomorphous, shortly pedicellate with the pedicel often sinuous; tepals 2 or 3, lanceolate, acute, alternate with the stamens; stamens 1–3, free or united at the base; filaments membranous, decurrent on the pedicel and cohering with the base of the ovary; anthers sagittate, introrse; pollen grains one-celled, ellipsoid, 3-colpate; ovary mostly perpendicular to the pedicel, 1-celled by reduction of the septum; one of the carpels smaller, almost free of the pedicel, without ribs or with 3–5 ribs, glabrous or with numerous small papillae in the apical part; the other carpel large, saucer-like, sometimes united at the base with the pedicel and perpendicular to the latter, and with the base of the filaments, provided

with 5 to 9 lighter coloured ribs, sometimes with minute papillae at the top; styles 2, filiform, unequal; placenta with 2 unequal lobes, attached by 2 short and thin stalks to the top and the base of the ovary; ovules relatively few. Fruit similar to the ovary, the smaller valve deciduous.
Type: *Castelnavia princeps* Tul. & Wedd. emend. Warming.
Distr.: 9 species in southeastern Brazil. (See map)

This genus is very sharply defined by the pronounced zygomorphy of the flower, a character that is not developed to such a high degree in one of the other American genera. It occurs, however, in the Asiatic genus *Farmeria*. The septum in the ovary disappears during the flowering period. The pollen grains are ellipsoid, 3-colpate, and they therefore agree with those found in the tribe *Lacideae* in the sense of ENGLER (1930). This character, in combination with some other ones compelled the present author in the first part of his study of the American Podostemaceae (1951, p. 12) to unite the tribes *Eupodostemoneae* and *Lacideae* of ENGLER to a single tribe *Eupodostemeae*.

The genus *Castelnavia* is subdivided by TULASNE and WEDDELL in two sections, viz. *Eucastelnavia*, comprising the species provided with a fastigiate base and the section *Castelnella*, in which the species with a linear base were brought together. Whether this subdivision may be regarded as natural, seems doubtful, as in *Castelnavia princeps* intermediate stages are found. But as there was too little material for study I had to leave this question undecided. The species themselves are quite easily distinguishable, though the lack of leaves in part of the material restricts the number of characters that can be used for the construction of a key to the species.

One new species is described, viz. *C. cuneifolia* which resembles *C. princeps* in its leaves, but differs from the latter in the shorter anthers and in the presence of 8 nerves instead of 10 in the ovary. The flower resembles that of *C. lindmaniana* and *C. serpens* in the lack of the small papillae at the top of the smaller valve. The leaves of the new species are pinnatilobed to pinnatipartite, while the leaves of the two species mentioned are repeatedly pinnate.

Geography (See map)

The genus seems to be confined to a comparatively small area; it has so far mainly been found in the Rio Araguay and its tributary the Rio Tocantin, where the species grow, according to WEDDELL in great abundance.

Key to the species:

1*a*. Stem distinctly widened, thalloid. Individuals solitary 2
b. Stems small or absent. Individuals springing from the flanks of narrow roots 6
2*a*. Smaller valve at the top with several small teeth 5
b. Smaller valve without teeth 3

3a. Both valves with 5 ribs 4
b. Smaller valve with 3 ribs; the other one without ribs . . . . 4. **C. lindmaniana** Warming
4a. Anthers 1-1.5 mm. Ovary with 10 ribs. . . . . 1. **C. princeps** Tul. & Wedd. em. Warming
b. Anthers 0.5-0.8 mm. Ovary provided with 8 ribs . . . . 5. **C. cuneifolia** v. Royen
5a. Stem c. 1 mm; tepals up to 0.25 mm; stamens 2-2.5 mm . . . . 2. **C. fimbriata** Tul. & Wedd.
b. Stem up to 0.5 mm; tepals c. 0.5 mm; stamens up to 1 mm. . . . . 3. **C. multipartita** Tul. & Wedd.
6a. Styles as long as or longer than the ovary 7
b. Styles shorter than the ovary . . . . . . 6. **C. serpens** Tul. & Wedd.
7a. Two stamens; smaller valve with 7 ribs 8
b. One stamen; smaller valve with 5 ribs . 7. **C. monandra** Tul. & Wedd.
8a. Styles 1.5-2 times as long as the ovary. Leaves 1-2 mm . . . . 8. **C. pusillima** Tul. & Wedd.
b. Styles 3-4 times as long as the ovary. Leaves 1-3 cm . . . . 9. **C. fluitans** Tul. & Wedd.

Section: EUCASTELNAVIA Tul. & Wedd.

Individuals solitary, with thalloid fastigiate base and distinct leaves. Flowers numerous, developing from cavities in the thallus—5 species in Central Brazil.

1. **Castelnavia princeps** Tul. & Wedd. emend. Warming (1882), Tul. (1849) 108; Tul. (1852) 164–166, t. 11 f. 1; Walpers (1852) 441; idem (1858) 789; Tul. (1863) 264–265, t. 76 f. 4; Weddell (1873) 80; Warming (1882) 79–89, 118–120, t. 13–15; idem (EP 1891) 22, f. 9; Engler (1930) 65, f. 57—Plate 4 f. 7–11.

Small, solitary herb, with a flattened base, the latter developing from a narrow area of attachment, repeatedly forked, ending in 2.5–6 cm long, flabelliform lobes with a membranous margin along which the leaves are inserted. Leaves 1.5–20 cm, a few times forked or pinnate, attached to the base with a slightly widened foot, ultimate divisions filiform, 0.5–4 mm. Flowers in cavities, which protrude with their margin above the thallus in the form of 2–3.5 × 1–2 mm large lobes, pedicel 1–3 mm, juvenile spathella ellipsoid, subsessile, membranous, with numerous papillae at the top, 3–4.5 × 1–2 mm, tepals 2, linear to spatulate, acute, 2.5–3.5 mm, one on either side of the andropodium and united with the latter above its zone of attachment with the ventral valve, in this way forming with the basal part of the andropodium a discoid organ which extends over the basal part of the dorsal valve, stamens 2 or 3, from 3–5 mm, filaments lanceolate, membranous, 2.5–4.5 × 0.2–0.8 mm, widened and united at the base and also with the basal part of the ventral valve, anthers sagittate, obtuse, 1–1.5 mm long, base of thecae obtuse, pollen grains 16 × 13 μ; dorsal carpel concave, 1 mm high, 2.5–3.5 mm long, 1.5–2.5 mm wide[1], cuneate at the base, provided with 5 ribs, ventral carpel

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[1] With high, long and wide of the carpels is meant in the case of the genus Castelnavia: the distance from the septae to the sides, the distance from top to base,

deeply concave, 2–3 mm long, 2–2.5 mm wide, 1.5–2 mm high, with undulate margin and united with the sinuous pedicel, provided with 5 ribs of which the marginal ones are indistinct, styles filiform to subulate, sometimes sinuous, 1–1.5 mm. Fruit similar to the ovary, with one persistent valve.
Type: Weddell s.n. in P.
Distr.: Brazil. (Provinces Goyaz, Minas Geraes and Rio Grande do Sul).

Goyaz — Rio Araguay, Weddell s.n., fl. fr. July (C, F, P); Rio Tocantin, Weddell 91, (B); Rio Uruhu, Glaziou 22014, fl. fr. July (C, P) — *Minas Geraes* — Caldas, Regnell III 1117, fl. fr. June (S, US); idem, Regnell III 2053, fl. fr. Febr. (C, P); idem, Regnell II 117 & II 1114, fl. fr. (P); idem, Mosén 1692, fl. fr. April (P); Dibaixo, Regnell s.n. (P); Rio Verde, Mosén 1694, fl. fr. April (S); Rio Capivary, Mosén 1695, fl. fr. May (S) — *Rio Grande do Sul* — Rio Bugres, without coll. 4, fl. fr. April (P).

2. **Castelnavia fimbriata** Tul. & Wedd., Tul. (1849) 108; Tul. (1852) 166–167, t. 11 f. 2; Walpers (1852) 441; idem (1858) 789; Tul. (1863) 265–266, t. 76 f. 1; Weddell (1873) 80–81—Plate 4 f. 12–16.

Very small herb with flattened, 2–3 cm wide, fleshy, flabelliform base with 5–10 × 2–3 mm large lobes with a membranous margin and passing into a few narrow, membranous, a few times forked, up to 12 mm long segments. Flowers in cavities in the lobes, juvenile spathella clavate, very thin, not or slightly protruding beyond the thallus; mature one infundibuliform, 1–1.5 mm long, pedicel 1–1.5 mm, tepals 2, linear, acute, one on either side of the andropodium, c. 0.25 mm, stamens 2–2.5 mm, filaments lanceolate, membranous, widened and united at the base, subcarinate, at the union obtusely saccate; anthers obtuse, c. 1 mm; pollen grains incompletely known; dorsal carpel shallowly concave, ovate, c. 1 mm long, up to 0.5 mm high at the top, with 5 ribs which are provided at the top with numerous small teeth; ventral carpel deeply concave, c. 1 mm wide and long, provided with 5 ribs; styles filiform, c. 1 mm. Fruit similar to the ovary, slightly exserted.
Type: Weddell s.n. in P.
Distr.: Brazil. (Province Goyaz).

Rio Araguay, Weddell s.n., fl. fr. July (P).

3. **Castelnavia multipartita** Tul. & Wedd., Tul. (1849) 110; Tul. (1852) 168–169, t. 11 f. 3; Walpers (1852) 442; idem (1858) 789; Tul. (1863) 266–267; Weddell (1873) 81; Warming (EP 1891) 22; Engler (1930) 65—Plate 4 f. 17–24.

Base 3–6 × 0.2–0.5 × 0.1–0.2 cm, repeatedly forked; lobes with membranous margin. Leaves linear to ovate-elliptic or spatulate, 2–5 × c. 1 mm, very thin, entire, inserted along the margin of the

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and the distance from side to side. This is done as the structure of the ovary differs from those known in the American part of the Podostemaceae.

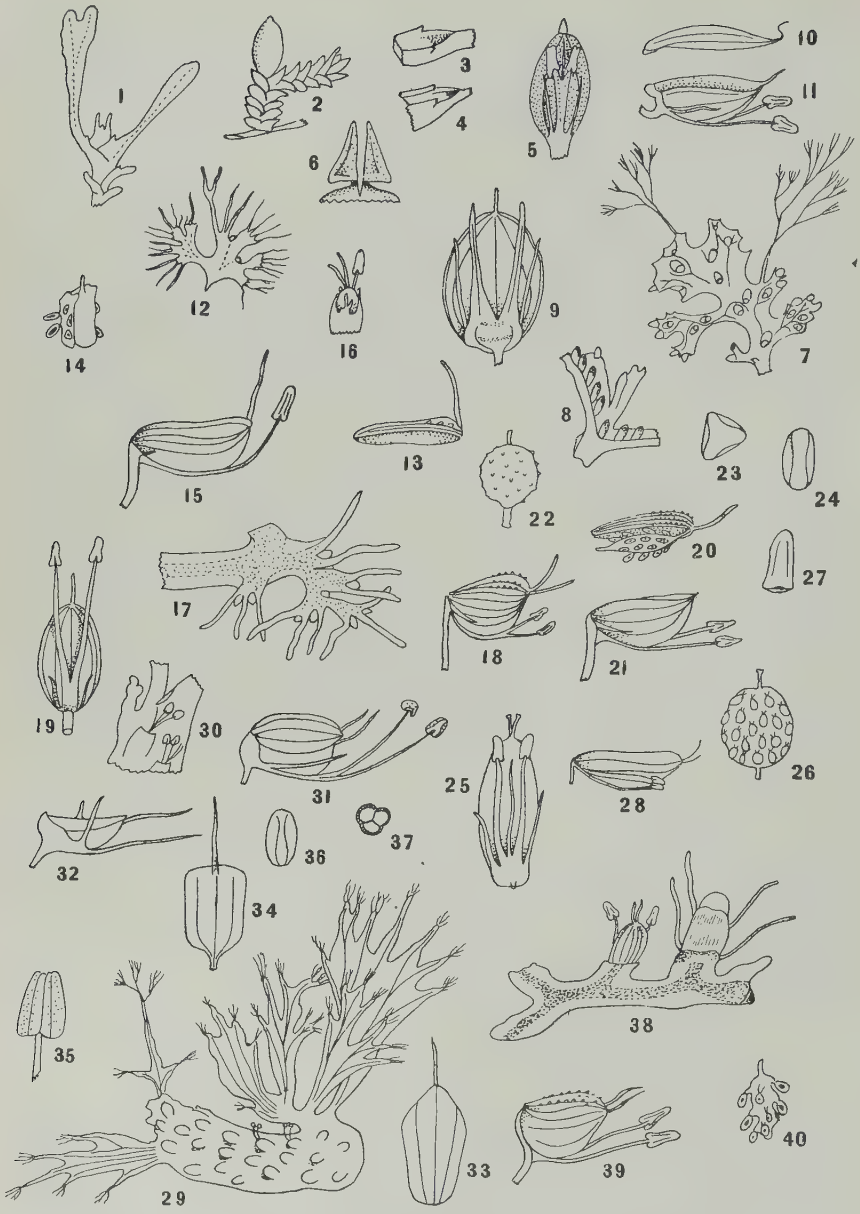

PLATE 4 — 1-6. **Podostemum galvone** Warming *(Puiggari 852)*, 1. apical part of shoot; 2. shoot with closed spathella; 3-4. basal part of petiole with stipule; 5. flower; 6. styles — 7-11. **Castelnavia princeps** Tul. & Wedd. *(Regnell III 1117)*, 7. habit; 8. base with buds; 9. flower; 10. smallest carpel; 11. largest carpel, with androecium — 12-16. **Castelnavia fimbriata** Tul. & Wedd. *(Weddell s.n.)*, 12. habit; 13. flower; 14. flower in spathella; 15. smallest carpel; 16. placenta — 17-24. **Castelnavia multipartita** Tul. & Wedd. *(Weddell s.n.)*, 17. habit; 18.

base. Flowers solitary, in the forkings, pedicel 0.8 mm, sinuous, juvenile spathella clavate, subacute, mature one infundibuliform, 1–2 mm, tepals 2, lanceolate, c. 0.5 mm, one on either side of the andropodium; stamens nearly 1 mm, filaments lanceolate, united at the base over a short distance, anthers very small, obtuse, pollen grains incompletely known, dorsal carpel ovate, conchiform, c. 1 mm long, provided with 7 ribs, of which the 2 marginal ones are indistinct, and with numerous small papillae at the top, ventral carpel deeply concave, c. 1 mm long. 0.5 mm high, at the base cohering with the pedicel and the base of the andropodium, which extends slightly over the top of the pedicel, provided with 9 ribs of which the marginal ones are indistinct, styles subulate, c. 0.5 mm. Fruit similar to the ovary.
Type: Weddell s.n. in P.
Distr.: Brazil. (Province Goyaz).

Rio Araguay, Weddell s.n., fl. fr. July (P).
This species was found to grow on wood, perhaps on a part of a root of a overhanging tree.

4. **Castelnavia lindmaniana** Warming (1899) 140–143, f. 35–37—Plate 4 f. 25–28.

Base irregular in outline, branched, thalloid, c. 3.5 × 2 × 0.3 cm. Leaves inserted along the margin at the end of the lobes, up to 18 cm, pinnate, the pinnae repeatedly forked, the ultimate divisions fasciculate, filiform, acute, up to 2 mm long (or more ?). Flowers known in the bud stage only, enclosed in the thalloid base, alternating with the leaves, subsessile, spathella clavate slightly constricted in the upper half, up to 4 mm, mature one unknown, tepals 3, lanceolate, acute, up to 2.5 mm, united at the base with the filaments, stamens 2, up to 3 mm, united at the base with the tepals, with the top of the pedicel and the base of the ovary, filaments lanceolate, up to 2 mm, anthers ovoid, obtuse or emarginate, base slightly incised, base of thecae obtuse, pollen grains incompletely known; ovary obovoid, obtuse, narrowed towards the base, obtusely triangular in transverse section, up to 2.5 × 1.5 mm, smaller carpel with 3 ribs, the larger one without ribs. Fruit unknown.
Type: Lindman A 2957 in S.
Distr.: Brazil. (Province Mato Grosso).

Serra do Itapirapuan, near Diamantino, fl. May (S).

5. **Castelnavia cuneifolia** v. Royen, nov. sp.—P. 259 and plate 4 f. 29–37.

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flower; 19. flower from underneath; 20. smallest carpel; 21. largest carpel with androecium; 22. placenta from above; 23-24. pollen grains — 25-28. **Castelnavia lindmaniana** Warming *(Lindman A 2957)*, 25. flower; 26. placenta from above; 27. anther; 28. flower, lateral view — 29-37. **Castelnavia cuneifolia** v. Royen *(Burck 9122)*, 29. habit; 30. base with flowers; 31. flower; 32. large carpel with androecium; 33. large carpel from beneath; 34. small carpel from above; 35. stamen; 36-37. pollen grains — 38-40. **Castelnavia serpens** Tul. & Wedd. *(Weddell s.n.)*, 38. habit; 39. flower; 40. placenta.

Base small, irregularly shaped, thalloid, branched or unbranched. Leaves along the margin of the base, cuneate to lanceolate, lobed to partite, 1–2.5 × 1 cm, papyraceous, distinctly palmatinerved; lobes lanceolate to triangular, at the top divided into subfiliform, branched segments; ultimate divisions lanceolate, c. 1 mm long, obtuse. Flowers numerous, in cavities in the thalloid base, pedicel very short, c. 1 mm, spathella clavate to campanulate, obtuse to umbonate, c. 3 mm, tepals 2, lanceolate, 2–2.5 mm, acute, one on either side of the andropodium, stamens 2, from 3–6 mm, filaments lanceolate, cohering at the base with the pedicel and the basal part of the larger carpel, up to 5 mm, anthers ovoid to cordate, obtuse, c. 0.8 mm, base emarginate, base of the thecae obtuse, pollen grains ellipsoid, 3-colpate, 16.6 × 11.4 μ; smaller carpel with 5 ribs, but the marginal ones indistinct, larger carpel with 5 ribs, but the marginal ones indistinct, up to 2 × 1.5 mm, styles subulate, 1.5–2 mm, free, unequal. Both valves of the fruit with 5 ribs.

Type: Burckell 9122 in W.

Distr.: Brazil. (Province Goyaz).

Near Sao Jeao do Araguay, Burckell 9122, fl. fr. (K, W).

Section: CASTELNELLA Tul. & Wedd.

Individuals either with a tubular base or provided with a distinct stem, arising along linear roots, and provided with well-developed leaves. Flowers one to numerous—4 species in Central Brazil.

6. **Castelnavia serpens** Tul. & Wedd., Tul. (1849) 109; Tul. (1852) 169–171, t. 12 f. 2; Walpers (1852) 441; idem (1858) 789; Tul. (1863) 267–268, t. 76 f. 5; Weddell (1873) 81; Engler (1930) 65, f. 57—Plate 4 f. 38–40.

Very small, up to 1 mm high individuals scattered along branched 1–1.5 mm wide, 5–10 mm long roots with an obtuse top. Leaves distichous, 1.5–4 mm, linear, entire, obtuse, at the base united to a membranous tube which opens with a ventral slit (? always), each individual with one flower, pedicel c. 1 mm, juvenile spathella clavate, membranous, slightly exserted, mature one tubuliform, 1.5–2 mm, tepals absent, stamens 2, from 2–2.5 mm, united at the base over a short distance, the andropodium united with the basal half of the ovary and decurrent on the pedicel, anthers obtuse, 0.5–1 mm, twisted when dry, base of thecae obtuse, pollen grains known in an incomplete state only, smaller carpel conchiform, subtruncate at the top, c. 0.8 × 0.5 mm, provided with 5 ribs, with numerous small papillae at the top, larger carpel deeply concave, c. 0.8 × 0.5 mm, obtuse, provided with 7 ribs, decurrent on the pedicel; styles filiform, c. 0.5 mm. Fruit similar to the ovary.

Type: Weddell s.n. in P.

Distr.: Brazil. (Province Goyaz).

Rio Araguay, Weddell s.n., fl. fr. July (P).

7. **Castelnavia monandra** Tul. & Wedd., Tul. (1849) 109; Tul. (1852) 171–173, t. 12 f. 1; Walpers (1852) 441; idem (1858) 789; Tul. (1863) 268, t. 76 f. 2; Weddell (1873) 81; Engler (1930) 65, f. 57—Plate 5 f. 1–5.

Very small individuals along membranous, 1 mm wide, branched roots; each individual provided with 4 to 6, distichous, linear, 5–20 mm long leaves. Basal leaves shorter than the apical ones, all widened and united at the base into a 1–1.5 mm long, 0.5–1 mm wide tube, which is shortly pubescent without, and develops a single flower. Pedicel 0.5-1 mm, juvenile spathella clavate, mature one tubuliform, 2–4 mm, tepals absent, stamen 1, 6–8 mm, filament filiform, 5–6 mm, at the base united with the ovary, decurrent on the pedicel, anthers obtuse, c. 0.6 mm, base of thecae obtuse, pollen grains incompletely known, smaller carpel elliptic, obtuse, 0.7–1 × c. 0.5 mm, provided with 5 ribs, but the marginal ones indistinct, with small papillae, the latter numerous at the top and diminishing in number towards the pedicel, larger carpel deeply concave, 0.5–0.8 × c. 0.5 mm, provided with 7 ribs, but the marginal ones distinct, styles linear, membranous, 1.5–2 mm. Fruit similar to the ovary.
Type: Weddell s.n. in P.
Distr.: Brazil. (Province Goyaz).

Rio Araguay, Weddell s.n., fl. fr. July (P).

8. **Castelnavia pusillima** Tul. & Wedd., Tul. (1849) 109; Tul. (1852) 174–175; Walpers (1852) 442; idem (1858) 790; Tul. (1863) 269–270; Weddell (1873) 81–82; Engler (1930) 65—Plate 5 f. 6–12.

Very small individuals along branched, compressed roots. Leaves 1–2 mm (according to Tulasne 6–7 mm), linear, widened at the base and united there into a minute thallus. Flowers between the leaves, pedicel 0.5–1 mm, juvenile spathella clavate, mature one campanuliform, 1–2 mm, tepals absent, stamens 2, from 2–3 mm, filaments lanceolate, 2–2.5 mm, membranous, over a short distance united with each other, with the basal part of the larger carpel and decurrent on the pedicel, anthers ovoid to obovoid, acute, base of the thecae obtuse, smaller carpel patelliform, 0.5–1 mm, provided with 5 ribs of which the marginal ones are indistinct, and with numerous small papillae especially at the top, larger carpel urceolate, 0.5–0.7 mm long, provided with 9 ribs of which the marginal ones are indistinct, at the base over a short distance united with the pedicel, styles filiform, 1–1.5 mm. Fruit similar to the ovary.
Type: Weddell s.n. in P.
Distr.: Brazil. (Province Goyaz).

Rio Araguay, Weddell s.n., fl. fr. July (P).

9. **Castelnavia fluitans** Tul. & Wedd., Tul. (1849) 109; Tul. (1852) 173–174; Walpers (1852) 441; Walpers (1858) 790; Tul. (1863) 269; Weddell (1873) 32; Engler (1930) 65—Plate 5 f. 13–16.

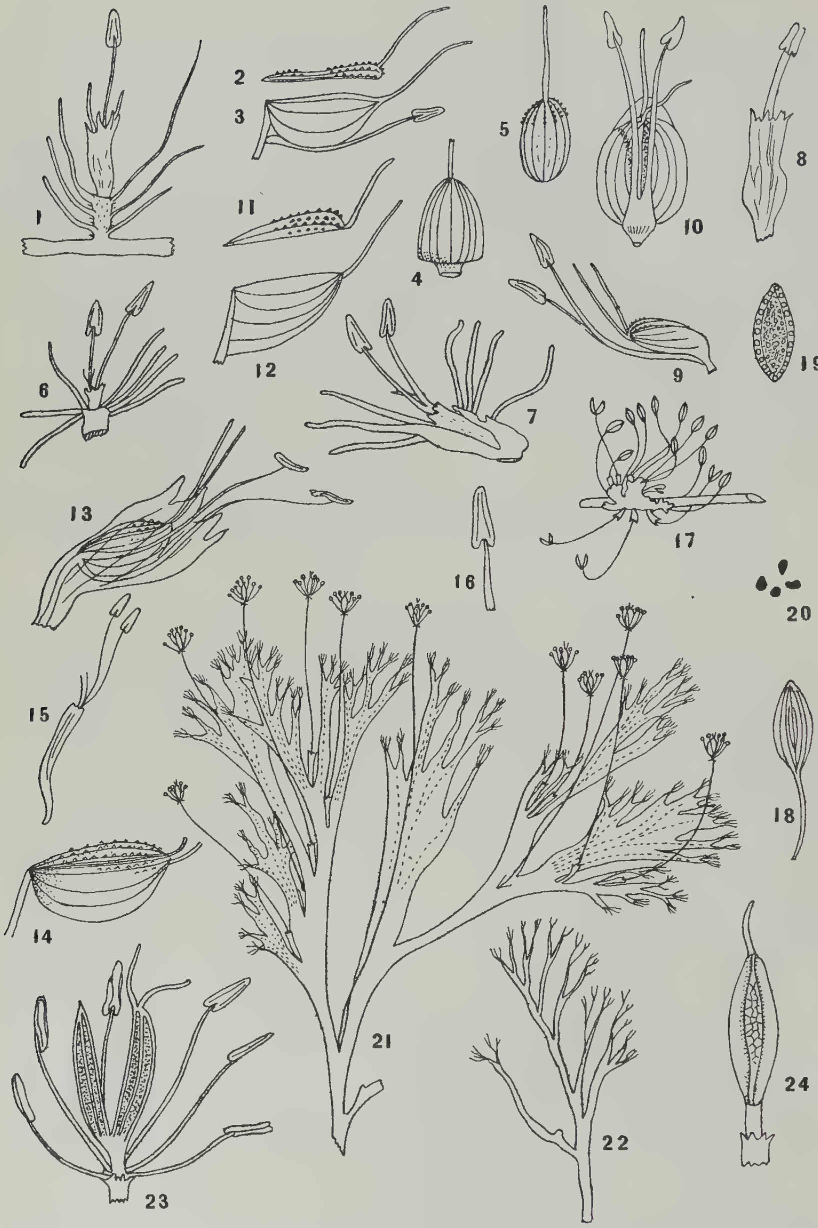

PLATE 5 — 1-5. **Castelnavia monandra** Tul. & Wedd. *(Weddell s.n.)*, 1. habit; 2. smallest carpel; 3. largest carpel with stamen; 4. smallest carpel from above; 5. largest carpel from beneath — 6-12. **Castelnavia pusillima** Tul. & Wedd. *(Weddell s.n.)*, 6-7. habit; 8. flower in spathella; 9-10. flower; 11. smallest carpel; 12. largest carpel — 13-16. **Castelnavia fluitans** Tul. & Wedd. *(Weddell s.n.)*, 13-15. flower, different views; 16. stamen — 17-20. **Blandowia striata** Willdenow *(redrawn after the picture made by Willdenow)*, 17. habit; 18. fruit; 19. placenta; 20. seeds — 21-24. **Apinagia aripecuruensis** v. Royen *(Ducke 15025)*, 21. habit; 22. leaf; 23. flower; 24. inner side of flower.

Small individuals formed along thin roots, almost stemless or the stem widened and forked (?). Leaves up to 3 cm, entire or divided into a few subfiliform segments, widened at the base and here shortly united into a small thallus. Flowers in fascicles of 2 to 4, arising between the leaves, pedicel 1–2 mm, sinuous, juvenile spathella clavate, mature one infundibuliform, 4–5 mm, tepals absent, stamens 2, from 7 to 8 mm, filaments filiform-linear, 6–7 mm, over a short distance united at the base and with the basal part of the ventral carpel and the top of the pedicel, anthers sagittate, c. 1 mm, obtuse, base of thecae obtuse, pollen grains known in an incomplete state only, smaller carpel elliptic, concave, 1–1.5 mm long, 0.5 mm high or less, provided with 7 ribs of which the marginal ones are indistinct, with numerous small papillae especially at the top, larger carpel deeply concave, 1–1.5 mm long, c. 1 mm high, with 9 ribs of which the marginal ones are indistinct, with some small teeth along the margin, styles compressed, linear, 4–4.5 mm. Fruit similar to the ovary.
Type: Weddell s.n. in P.
Distr.: Brazil. (Province Goyaz).

Rio Araguay, Weddell s.n., fl. fr. July (F, P).

EXCLUDED SPECIES

1. **Castelnavia warmingiana** Glaziou, (1911) 576, nomen nudum.

This is no Castelnavia, but an Apinagia. However, as the type-specimen consists of fruits and leafless stems only, I am not able to identify it. The equal carpels are a distinct feature for Apinagia and are never found in Castelnavia.
Type: Glaziou 22004 in P.
Distr.: Brazil. (Province Goyaz).

Rio Macaco, Glaziou 22004, fr. June (B, K, P).

III DUBIOUS GENERA

1. **BLANDOWIA** Willdenow

Willdenow (1809) 100–101, t. 4 f. 2; Weddell (1873) 85; Engler (1930) 68—*Dicraea* Du Pet. Th., § Blandowia Tul. (1849) 101; Gray (1849) 419–420—*Apinagia* Tul., § Blandowia Tul. (1852) 106–108.

The original description given by Willdenow is: Capsula bivalvis bilocularis. Semina dissepimento oblongo affixa.
Type: Blandowia striata Willdenow.
Distr.: Chili.

Willdenow's description of the genus is rather meagre, but the material does not permit of giving more details. Until not only one species is known, viz. *B. striata*, and of this species only fruits and an incomplete base are present. The material is according to the drawings and the description so poor that I am unable to decide whether it really is a *Podostemacea*, though I must admit that it has a distinct podostemaceous habit. Willdenow's statement that this species lives on trees, makes its taxonomic position even more dubious.

Sturm in 1835 described under the name *B. striata* another species from Italy living on trees and in moss-cushions. Baroni in 1900 proved that this species was *Targionia epiphylla*, a liverwort. As the name *B. striata* was used by Sturm for a different species, Tulasne in 1852 correctly changed the name used by the latter; he called it *Apinagia preissi*. However, not only this species was given another name, but the type-species, *B. striata* from Chili received also a new name, viz. *Dicraea willdenowii* Tul. as Tulasne (1849) regarded this species as belonging to *Dicraea*. Moreover a new species of *Lacis* described by Bischof (1835) under the name *L. fallacissima* was recognized by Tulasne as conspecific with *Blandowia striata*. In 1905 Nash placed *Neolacis myriophylla* Weddell in this genus *Blandowia*, but this is obviously a mistake as it is clear that it belongs to *Marathrum*, in fact it is conspecific with *M. tenue* Liebmann.

1. **Blandowia striata** Willd. (1809) 100–101, t. 4 f. 2; Sprengel (1827) 139 & 236; Engler (1930) 68—*Lacis fallacissima* Bischof, (1835) 963—*Dicraea willdenowii* Tul. (1849) 101; Gay (1849) 419–420—*Apinagia ? willdenowii* (Tul.) Tul. (1852) 106 —*Apinagia striata* Grisebach p.p. in msc.—Plate 5 f. 17–20.

As I have not seen the material I must confine myself to reproducing Willdenow's description:

Frons plana depressa lobata laevis facie Collemae, lobis adscendentibus obtusis. Perichaetium tubulosum breve lacerum. Seta filiformis. Capsula elliptica striata bivalvis ab apice ad basin dehiscens, bilocularis. Dissepimentum contrarium oblongum, post dehiscentiam capsulae deciduum. Semina oblonga brevi pedicello margini dissepimenti affixa.

Type: Lehmann s.n. in B, duplicates in P and C.
Distr.: Chili.

EXCLUDED SPECIES

Blandowia preissii (Tul.) Wedd., (1873) 85; Engler (1930) 68—*Apinagia ? preissii* Tul. (1852) 108—*Blandowia striata* Corda in Sturm (1805) 107–116, t. 33 non Willdenow.

As Baroni has shown, this species is identical with the hepatic *Targionia epiphylla*.

2. CARAJAEA (Tul.) Wedd.

As I have not seen the material I reproduce here the description given by Weddell:

Flores hermaphroditi, nudi, angulis bifurcationum frondis solitarie impositi, erecti, singuli spathella urceolata primum clausa deinque apice pervia pro parte inclusi. Stamina 2, libera, unilateralia, filamentis planis elongatis seorsum attenuatis, antheris ovatis ore spathellae longiuscule exsertis. Staminodia nulla? Ovarium sphaericum, in fundo spathellae sessile, laeve. Stigmata 2, elongata, linearia, libera, exserta. Capsula non visa.
Type: *Carajaea orthocarpa* (Tul. & Wedd.) Wedd.
Distr.: Rio Araguay, Santa Maria falls, Brazil.

1. **Carajaea orthocarpa** (Tul. & Wedd.) Wedd., (1873) 85—*Castelnavia ? orthocarpa* Tul. & Wedd., Tul. (1849) 110; Tul. (1852) 175; Tul. (1863) 270.

Description according to Weddell:
Pusillima, fronde lineari dichotoma ramisque primariis membranaceo-spongiosis, ramulis filiformibus. Rami frondis vittiformes, plus minus elongati ramulique mire recti et textura firmiore ad dorsum lapidis cui arcte haerent magis minus inter se occursantes. Flores ad angulos bifurcationis frondis praesertim occurentes, spathella sessili late ovata, ore contracto acute 4–6 dentato. Stamina e fundo spathellae erecto-divergentia illamque dimidia longitudine superantia, antheris filamento circiter triplo brevioribus, conneccivo ultra loculos nonnihil protracto. Ovarium sessile, sphaericum, laeve, spathella omnino inclusum, stigmatibus ovarii diametrum longitudine nonnihil excedentibus acutus divergentibus in vivo pallide rubentibus oreque spathellae longiuscule exsertis.
Type: Weddell s.n. in P?
Distr.: Known from the type-locality only.
Rio Araguay, Santa Maria falls, Weddell s. n. (P?), fl. fr.

3. VELOPHYLLA Clarke

Durand (Index Gen. Phan., 1888, 364) refers to B. Clarke, 'A new arrangement of the Phanerogamous plants (1866), tab. 4', but this apparently is a mistake. Even with the expert cooperation of Mr Sandwith of the Kew Herbarium and of Dr Taylor of the British Museum, I was unable to trace this genus.

4. ARIADNEA Klotsch

This genus with one species *A. pectinata* Kl. is published in Schomburgk, 'Reisen in British-Guiana 3 (1848) 930' and is reported from the Essequibo, Mazaruni and Cuyuni river, but as no specimen are mentioned I was unable to trace this genus.

5. ARIORISTRA Klotsch

This genus with one species, *A. marathrioides Kl.*, is published on the same page as the genus Ariadnea and also here I was unable to trace the material.

IV. ADDITIONS AND CORRECTIONS TO PART I

Page

3. 7th line from below read: In 1933 Sprague . . .
5th line from below add after Engler (1926): Beitr. Fl. Afrika 52, Engl. Bot. Jahrb. 60 (1926) 453.
Read the last sentence as follows: According . . ., which existed already when the continents were connected to each other and birds could transport the seeds . . .
10. In the 8th line read: Bentham, i.s.o. Hooker.
13. Add in the 21st line: Macarenia.
22. Add in the 5th line from below: (p. 29).
23. Read in the 2nd line: solitary, i.s.o. fascicled.
25. Fl. in the 4th and fr. in the 5th line have to be changed.
29. Read in the 11th line: Von Chamisso, i.s.o. Schiede.
In view of some objections raised against the use of the name Apinagia Tul. the following can be added to the 2nd alinea. Von Chamisso in 1835 distinguished in the genus Lacis 3 sections and used these names as a generic name when describing the species, viz. Mourera, Marathrum and Lacis. Endlicher in 1837, also distinguished 3 sections, Mourera, Marathrum and Neolacis but in the genus Mourera. Lindley, finally, in 1846, mentions Neolacis as a genus but in the synonymy of Mourera. This name, therefore, can not be regarded as a valid publication of the genus Neolacis. Weddell in 1873 was the first who used Neolacis as a genus-name and who gave a description of it. But already in 1852 Tulasne described the genus Apinagia including the species of the section Neolacis of Lacis or Mourera of earlier authors. Therefore Apinagia has a priority over Neolacis of 24 years and not Neolacis over Apinagia of 6 years.
32. In the KEY read 2*a* as follows: Fruit and ovary with 10-14 ribs.
40. In Apinagia staheliana the fruit is provided with 2 long ribs and 4 shorter ones.
42. In the 9th line add after "Mussoemba-falls: Tresling 110.
52. In the 10th line from below delete: Plate 3 f. 8-13.
53. In the 5th line from below add: Plate 3 f. 8-13.
69. In the 15th line add after "unknown": but the description is extracted from that of Tulasne.
89. In the 6th line read Aitken and not Aitkers.
92. Add in the 15th line: and the three Guianas.
98. 3d line: delete fr.
102. Add to Rhyncholacis dentata: ultimate divisions mainly 0.2 mm long or shorter but sometimes up to 1.2 mm; tepals 0.5-1.5 mm; styles 1-3 mm.
117. Delete in the 25th line: Rio Guama, Huber 1815, Dec. (G.-Boiss.).
121. Add after 2. Jenmaniella jenmanii (Engler) van Royen, nov. comb.: Plate 14 f. 4-6.
131. In the 10th, 11th and 12th line change "Var." in "forma".
139. Change Aitkers in Aitken.
145. Delete after C. Sprengel: Linnaeus, Species Plantarum etc.
148. Apinagia intermedia Warming must be printed in italics.

PLATE

2. Delete: The scales are given in millimetres.
8. Add: 18-19. Marathrum minutiflorum Engler, forma minutiflorum (Rothschuh 411), 18. flower; 19. primary pinnae.

The following new collections came to my attention:

Apinagia flexuosa, van Royen (1951) 36 — British Guiana, Essequibo-river, Kurupukari-falls, N. Beccari 141, fl. fr. Nov. (FI, U).

Apinagia staheliana, van Royen, l.c. 40 — British Guiana, Berbice-river, Itabru-falls, near Marlissa, N. Beccari 139 & 140, fl. fr. Dec. (FI, U).

Apinagia tenuifolia, van Royen, l.c. 42 — Brazil, Cachoeira do Rio Branco, Kuhlmann 197, fl. fr. (CGE).

Apinagia richardiana, van Royen, l.c. 44 — British Guiana, Essequibo-river, Kurupukari-falls, N. Beccari 142, fl. fr. Dec. (FI, U).

Apinagia glaziovii, van Royen, l.c. 46 — Brazil, Cachoeira do Alta Ariramba, Ducke 14892, fr. Sept. (CGE).

Apinagia riedelii, van Royen, l.c. 47 — Brazil, Rio Taquary, Hoehne 1201 (3921?), fl. fr. May (CGE).

Apinagia exilis, van Royen, l.c. 53 — British Guiana, Essequibo-river, Kurupukari-falls, N. Beccari 152, fl. fr. Nov. (FI, U); Berbice-river, Marlissa-falls, N. Beccari 153, fl. fr. Dec. (FI, U).

Apinagia dissecta (Montagne) Engler (1930) 38; erroneously as the first author always Weddell has been regarded — *Apinagia dissecta* (Wedd.) Engler, van Royen, l.c. 68 — *Neolacis dissecta* Wedd. (1873) 62 – *Anthoceros dissectus* Montagne (1838) 38-57; Proskauer (1953) 65-67, f. 1.

By the courtesy of Dr Bor, editor of the Kew Bulletin, I have been allowed to consult a paper, which will not be published, by Dr Proskauer, Berkely, California on "Anthoceros" dissectus Montagne. Dr Proskauer draws the attention to this Anthoceros because he supposes that it must be a Podostemacea. In the Synopsis hepaticorum (1844) 590, this is already suggested by Gottsche, and Weddell in 1873 described it as a Neolacis species, unfortunately without referring to Montagne's publication. As I was unaware of this oversight I had used Weddel's name as the original author, but Dr Proskauer kindly drew my attention to Montagne's paper. However, it seems to me, that Weddell did not know this publication, as he quoted "Anthoceros dissectus Montagne, in msc". I here wish to express my sincere thanks to Dr Proskauer for his kind advice.

Apinagia nov. sp.?, van Royen, l.c. 69 — Brazil: Huber 1783 (CGE).

Marathrum minutiflorum, forma **indifferens,** van Royen, l.c. 81 – El Salvador, dept. San Salvador, Las Cataractes, Panchimalco, Fassett 28669, Jan. (WI, U).

Rhyncholacis hydrocichorium, van Royen, l.c. 96 — British Guiana, Essequibo-river, Kurupukari-falls, N. Beccari 144, fl. fr. Nov. (FI, U); Berbice-river, Marlissa-falls, N. Beccari 146, fl. fr. Dec. (FI, U); Itabru-falls, near Marlissa, N. Beccari 147, Dec. (FI, U).

Rhyncholacis palmettifolia, var. **rosea,** van Royen, l.c. 99 — British Guiana, Essequibo-river, Acuri-falls on top of Kurupukari-falls, N. Beccari 145, fr. Nov. (FI, U).

Rhyncholacis minor, van Royen, l.c. 102 — Brazil, Rio Guama, Cachoeira do Ourem, Huber 1792, fr. Dec. (CGE); Rio Capim, Huber 865, fl. fr. (CGE).

Rhyncholacis brevistamina, van Royen, l.c. 107 — British Guiana, Demerara, Great falls, N. Beccari 148, fl. fr. Sept. (FI, U).

Rhyncholacis jenmanii, forma **laciniata,** van Royen, l.c. 110 — British Guiana, Demarara-river, Canister-falls, N. Beccari 143, fr. Nov. (FI, U).

Jenmaniella fimbriata, van Royen, l.c. 122 — Brazil, Rio Guama, Cachoeira do Ourem, Huber 1806 (CGE).

Macarenia clavigera, van Royen, l.c. 126 — Colombia, San Juan de Arama, left bank of Rio Güejar near "Los Micos", alt. 500 m, Idrobo & Schultes 717, fl. fr. Dec. (COL, U, US); idem, Idrobo & Schultes 553, fl. fr. Dec. (COL, U, US). In this material the species is said to have red latex.

Apinagia aripecuruensis van Royen, nov. sp.—P. 260 and plate 5 f. 21–24.

Small, branched herb, 3–7 cm high; internodes terete to compressed, 0.5–1 cm long, 2–5 mm wide. Leaves 2–5 cm long, at the base 1–5 mm wide, repeatedly forked or flabelliform with repeatedly forked segments, palmatinerved with prominulous ribs, membranous; ultimate segments narrowly linear, acute, nerveless, 1.5–3 mm long. Flowers solitary, pedicel 1.5–5.5 cm long, provided with two wings, tepals 7–9, linear, acute, c. 0.5 mm long, stamens 7–9, in a complete whorl, 3–4.5 mm long, anthers sagittate, 1–1.5 mm long, mucronate, truncate or emarginate at the top, emarginate at the base, base of thecae mucronate, obtuse or emarginate; pollen grains ellipsoid, 3–colpate, 16 × 12 μ; ovary ellipsoid, 2–3.5 × c. 1 mm, subobtuse at the top, borne by a 0.5–1 mm long, terete, gynophore and provided with 6 or 8 prominent ribs; the ribs on the sutures thin and closely approaching each other or absent; styles 2, from 1–2 mm long, subulate or, sometimes, ribbon-like flattened, obtuse, papillate. Fruit similar to the ovary, each valve provided with 3 or 5 ribs.

Type: Ducke 15025 in CGE, duplicate in U.

Distr.: Rio Eripecurú, Cachoeira do Inferno, fl. fr. Oct. (CGE, U).

This species resembles *Apinagia digitata* in its leaves but has no tufts of filaments on the latter. From *Apinagia exilis* it differs in the 6 or 8 long ribs, while in *A. exilis* which closely resembles this species, there are 6 short ribs. From the other Apinagia species it differs in the distinct gynophore. This character was only known until now in the genus Jenmaniella and *Apinagia divertens*, thus obscuring the limits between Jenmaniella and Apinagia.

V. LATIN DESCRIPTIONS OF THE NEW TAXA

Oserya minima van Royen — Cf p. 220 et tab. 1 f. 8-11.

Individua pusilla, subopposita vel opposita secundum radices tenues, ramosas, applanatas; basi thalloidea vel tubuliformi. Foliis 1 - 3 × furcatis, ad 5 mm longis, laciniis angustissimis. Floribus solitariis, pedicello circa 1 mm longo; spathella juvenili clavata, obtusa, sessili, robusta, basem thalloidam paulo excedente, matura tubuliformi; tepalis 2 vel 3, duobus in latera, tertio in dorsum filamenti inserto, interdum lateralium altero absente, c. 0.3 mm longis; stamen 1; filamento subulato; antheris obtusis vel emarginatis; basi ad medium vel minus incisis; thecis interdum inaequalibus; lobis obtusis, basifixis, extrorsis, circa 0.5 mm longis; granulis pollinis ellipsoideis; ovario oblique ellipsoideo vel globoso, obtuso, basi attenuato, 8-costato, circa 1.5 mm longo; carpellis 2 inaequalibus; stylis brevibus, paulo papillatis, cylindricis, obtusis, basi cohaerentibus, c. 0.3 mm longis. Fructu maturo ignoto.

Typus: *Went, s.n., in fluvio Surinamei prope Kabelstation, Suriname, in herb. U.*

Hac species Oseryae flabelliferae affinis est, sed differt tepalis 3 filiformibus et ovario 8-costato. Descriptio Oseryae sphaerocarpae in paucis singulis cum descriptione supra data convenit, propter materiam im probam O. sphaerocarpae junctionem duarum specierum hacum incertum est.

Podostemum ceratophyllum Michaux, var. **circumvallatum** van Royen — Cf p. 235.

A var. ceratophyllo differt caulibus brevioribus, cicatricibus in caulis parte basali congestis et numero dioribus, stipulis obtusis, caulibus rigidis et scabris. Flores et fructus ignoti.

Typus: *Standley 56080 in Honduras collectum, in herb. US.*

Podostemum dimorphum van Royen — Cf p. 235 et tab. 2 f. 14 - 16.

Caules steriles a fertilibus separati. Caulis sterilis 20 - 60 cm altus, simplex, laxus, interdum subalatus, compressus. Folia bipinnata, 2 - 12 cm longa, rachi tereti vel subtereti, pinnis pinnatis vel furcatis, segmentis ultimis linearibus, 2 - 6 mm longis; stipula cum basi lata inserta, concava, membranacea, apice integra vel bifida. Specimina fertilia acaula vel in caulem 0.5 - 3 mm altum producta, efoliata, basi conspicue elata et lobata, caulibus suboppositis in radicibus compressis, uniflora. Flores in alabastro solum cogniti, spathella clavata, mammillata, rigida, scabriuscula; tepala 3, linearia usque filiformia, acuta, circa 1 mm longa, tepalo tertio in filamentorum bifurcatione, quam altera dua paulo breviore; andropodium 4-angulatum, membranaceum, circa 1 mm longum, filamentis circa 0.8 mm longis, membranaceis, apice dilatatis, antheris irregulariter obtusis; granulis pollinis 2-

loculatis, ceterum incomplete cognitis; ovarium globosum, incomplete cognitum; styli filiformes, acuti, 0.5 mm longi. Fructus ignotus.

Typus: *Dusén 16540, in prov. Parana, Brasilia, collectum, in herb. U.*

Haec species facile distinguenda est anthesi differente et individuis fertilis nati ex eadem radice. Stipulae eisdem P. ceratophyllae similes, sed folia segmentis ultimis longioribus et latioribus.

Podostemum undulatum van Royen — Cf p. 236 et tab. 2 f. 20.

Individua parva, subramosa, 2 - 5 cm alta, opposita in radicibus tenuibus, circa 1 mm latis; internodia subcompressa, flexuosa, fragilia. Folia paucifurcata, 2 - 7 cm longa, petiolo subtereti, basi triangulari, subdecurrente, segmentis ultimis spatulatis, subacuminatis, distincte uninerviis, membranaceis, marginibus undulatis; stipulae concavae, 1 - 2 mm longae, subamplexicaulae, apicibus 1 vel 2, brevibus, acutis; stipulae foliorum apicalium apice integrae, basales bifidae. Flores pauci, terminales, in alabastro tantum cogniti, pedicello ignoto,spathella juvenili clavata, acuta, matura ignota; tepala 3, filiformia, circa 1 mm longa, andropodio circa 0.5 mm longo, membranaceo, filamentis 0.5 mm longis, apice dilatatis, antheris thecibus inaequalibus, apice obtusis vel emarginatis, thecis basi obtusis; granulis pollinis 2-loculatis, incomplete cognitis; ovarium ellipsoideum, 8-costatum, sed e numero costarum 2 valde approximatis; styli filiformes (?), basi coherentes. Fructus ignotus.

Typus: *Ule 804, in Brasilia collectum, in herb. P.*

Haec species P. ceratophyllo et P. aguirensi foliis et stipulis similis, sed differt ab iis segmentis ultimis spatulatis et longioribus. Etiam P. schenckii et P. osteniano similis sed differt ab specie priori segmentis ultimis abrupte elatis et ab specie posteriori segmentis ultimis latioribus insuper nervi munitis.

Var. **angustifolium** — Cf p. 237.

Differt a var. undulato magnitudine, segmentis ultimis angustioribus, spatulatis, stipulis longioribus, 1 - 3 mm longis. Caulis 3 - 12 cm altus, folia 5 - 8 cm.

Typus: *Jörgensen 4989, in Paraguay collectum, in herb. C.*

Podostemum dentatum van Royen — Cf p. 241 et tab. 3 f .15 - 19.

Individua parva leviter ramosa vel simplicia, subopposita vel opposita secundum radices tenues. Caulis 2 - 10 mm longus. Folia 0.5 - 2.5 cm longa, integra (vel bifida?), basi 0.5 - 2 mm longa, oblique inserta, abrupte in laminam linearem uninerviam attenuata; stipula triangularis vel ovoidea, acuta, cum basi angusta, lineari ad folii basem mediam inserta; apice in foliis superioribus dente brevi triangulari, 0.5 mm longo munita. Cicatrices basi spatulatae. Flores pauci, terminales et axillares, pedicello 1 - 3 mm longo; spathella juvenilis clavata, umbonata, coriacea, maturitate campanuliformis; tepala nota 2, linearia, acuta, tertium ignotum; andropodium membranaceum, filamentis lanceolatis, 0.5 mm longis; antherae sagittiformes, basi et apice profunde emarginatae, thecis inaequalibus apicibus acutis et basibus subacutis ad acutis; granulis pollinis 2-loculatis, incomplete cognitis; ovarium ellipsoideum ad ovoideum, costis 8, indistinctis, latis, compressis; styli filiformes, 0.5 - 0.8 mm. longi. Fructus bivalvus, valva altera costis 5 erectis vel subcurvatis munita, altera caduca, costis 5 leviter S-formibus munita.

Typus: *Ule 1876, in Brasilia collectum, in herb. P.*

Haec species P. galvoni similis denti in petioli parte superiori, sed differt petiolo abrupte contracto apud speciem posteriorem, stipulis longioribus et statura minori.

Castelnavia cuneifolia van Royen — Cf p. 249 et tab. 4 f. 29 - 37.

Individua parva, acaula, basi irregulariter thalloidea, ramosa vel simplicia. Folia marginalia, cuneata ad lanceolata, pinnatilobata ad pinnatipartita, papyracea, distincte nervosa, palmatinervia; lobi lanceolati ad triangulares apice in lacinias subfiliformes ramosas divisi, segmentis ultimis lanceolatis, circa 1 mm longis, obtusis. Flores numerosi, in basi thalloidea e cavitatibus erumpentes, pedicello brevissimo circa 1 mm longo, spathella clavata ad campanulata, obtusa ad umbonata; tepala 2, lanceolata, acuta; stamina 2, 3 - 6 mm longa, filamentis lanceolatis, basi in pedicellum decurrentibus et cum carpelli maioris parte basali conjunctis, usque ad 5 mm longis; antherae ovoideae usque cordatae, obtusae, basi emarginatae, thecarum basi obtuso; granulis pollinis ellipsoideis, 3-colpatis; ovarii carpellum minus 5-costatum, costa marginali indistincta, usque 2 mm longum; styli subulati, 1.5 - 2 mm longi, liberi, inaequales. Fructus valvae ambae 5-costatae.

Typus: *Burckell 9122, in Brasilia collectum, in herb. W.*

Haec species C. principi similis foliis, sed differt antheris brevioribus et nervis 8, non 10, in ovario. Flos eo C. lindmanianae et C. serpentis similis papillis parvis carentibus ad apicem valvae minoris. Folia C. cuneifoliae pinnatilobata usque pinnatipartita ex contrario folia specierum duarum iteratim pinnata.

Apinagia aripecuruensis van Royen, nov. sp. — Cf p. 257 et tab. 5, f. 21 - 24. Herba parva ramosa, 3-7 cm alta; internodia teretia ad compressa, 0.5-1 cm longa, 2-5 mm lata. Folia 2-5 cm longa, basi 1-5 mm lata, iteratim furcata vel flabelliformia, segmentis iteratim furcata, palmatinervia, costis prominulis membranacea; segmenta ultima anguste linearia, acute, enervia, 1.5-3 mm longa. Flores solitarii; pedicellus 1.5-5.5 cm longus, alis duobus munitus; tepala 7-9, linearia, acuta, circiter 0.5 mm longa; stamina 7-9, in verticillam completam posita, 3-4.5 mm longa; antherae sagittatae, 1-1.5 mm longae, apice mucronatae, truncatae vel emarginatae, basi emarginatae; thecarum basis mucronata, truncata vel emarginata; pollinia ellipsoidea, 3-colpata, 8μ alta, 6μ diam; ovarium ellipsoideum, 2-3.5 mm altum, circiter 1 mm diam, apice subobtusum, gynophoro tereti, 0.5-1 mm longo sustentum et costis prominentibus 6 vel 8 munitum; costae in suturis tenues et valde se approximatae vel desunt, styli 1-2 mm longi, subulati vel interdum applanati, lineati, obtusi, papillati. Fructus ovario similis, utraque valva costis 3 vel 5 munita.

Typus: Ducke 15025, in Brasilia collectum, in herb. CGE.

Haec species Apinagia digitata foliis similis, sed differt foliis fasciculis filamentorum destitutis. Differt ab Apinagia exilis costis longis 6 vel 8: in Apinagia exilis ad 6 costae breves sunt. Differt ab Apinagiae speciebus ceteris gynophoro conspicuo. Hic character usque adhuc in generi Jenmaniella et in Apiniagia divertens solum cognitus, ergo obscurans delimitationes inter Jenmaniellam et Apinagiam.

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VII. LIST OF COLLECTORS' NUMBERS

The collectors' numbers and the pages are printed in *italics*; the numbers in parentheses are the pages on which the collectors' numbers are cited. 'S.n.' means unnumbered specimen.

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THE REGULATING INFLUENCE OF TRANSPIRATION AND SUCTION TENSION ON THE WATER AND SALT UPTAKE BY THE ROOTS OF INTACT VICIA FABA PLANTS

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(*Received April 1st* 1954)

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Chapter I

GENERAL INTRODUCTION

Though the processes of water uptake by the root and water transport through the plant are well known in general lines, little attention is paid to an important factor in these processes, viz. the resistance of the root tissue to the water current. Renner (1929) showed clearly that this resistance is considerable. Koehnlein (1930), Kramer (1933) and Brewig (1937) corroborated his observations. The most striking fact is, that this resistance is not a constant one but depends on various circumstances. Jost (1916) was the first physiologist to find that an increase in water uptake was not proportional to the increase in suction tension in the xylem vessels. He observed that at increasing suction tensions the water uptake per atmosphere pressure difference, increased. This can be explained in two different ways.

Firstly, it is possible that with a greater water deficit in the plant a considerable quantity of water is taken up by an active water pumping mechanism. The other possibility may be that in this case the resistance of the root tissue against the water current diminishes.

KOEHNLEIN (1930) observed that at stronger suction the suction tension in the vessels was more effective but that it was not possible to get the same efficiency when the outer solution was at 0° C. He suggested, therefore, that the first possibility mentioned by JOST was the right one. An active water uptake mechanism strengthens the effect of the mechanical suction. At 0° C this mechanism comes to a standstill and the root system behaves like a pure physical system.

BREWIG (1937) who elaborated a method of precisely determining the resistance of the various root zones, showed that the latter interpretation of JOST was the more likely one. It appeared that at low suction tension in the xylem vessels the resistance to the water current was greatest in the basal region and smaller in the zones near the tip. On enhancing the transpiration the resistance of the apical zones remains unchanged but the resistance of the basal root parts decreases and becomes as small as or smaller than the resistance in the apical zone.

Little attention was paid to these observations in the literature, probably because of the fact that no explanation of these changes in resistance could be given. The author was able to corroborate the results of Brewig's experiments completely. Using an improved method more regular results were obtained.

Moreover, it appeared possible to get some insight into the causal relations of the changes in resistance. In view of the importance of these changes in resistance to the water current for transpiration and salt uptake, a summary of the results of the preceding experiments is given in Chapter III.

The results of SIERP and BREWIG (1935), BREWIG (1936 cf) and BROUWER (1953*a*) confirmed the fact that the root of *Vicia faba* was permeable to water over a great length, at any rate up to 18 cm from the root tip. This seems contrary to the conceptions of SCOTT and PRIESTLEY (1928) and SCOTT (1928) who on account of anatomical data confined the water uptake to the apical 5 cm region, but it agrees with the experiments of POPESCU (1926), HÖHN (1934) and others who showed that the roots of various plants were able to absorb water over their whole length.

Though the methods used had some drawbacks (in fact all parts of the root, with exception of the tested one, have been prevented from the water uptake) it was clear that the capacity to absorb water was not limited to the apical region.

It is a surprising fact that, up to now, it is unknown which zone of the root of an intact plant shows the maximum salt uptake. Most experiments on this problem have been performed with excised roots or root systems. The experiments of PREVOT and STEWARD (1936) show nothing but the binding in the root tissue. It is not surprising that this binding capacity is most important in the root zone which shows the most rapid growth. The experiments of LUNDEGÅRDH (1945) deal with the same process and also the transfer to the vessels is determined. It is, however, a special uptake, viz. the uptake of an excised root (bleeding). Kramer and Wiebe (1952) used intact plants but determined only that portion of the total uptake that has been withheld by the root tissue and this is nothing but the accumulation of ions as shown in the experiments of Prevot and Steward.

The experiments described below show the uptake of the different zones of the root of an intact plant. The method used makes it possible to vary the water uptake of the tested root and in this way the problem of the influence of the water uptake on the salt uptake is drawn into the research. The anion uptake of the different zones can also be traced at different rates of water uptake.

There are few other problems in plant physiology that have attracted as much attention as this during the last 50 years. In spite of this no agreement has been reached as yet.

At first plant physiologists assumed that salts were taken up and transported passively by the transpiration stream. This concept could only be maintained if the amount of ions taken up was directly proportional to the amount of water absorbed. The experiments of Hasselbring (1914), Kiesselbach (1916), Mendiola (1922), Muenscher (1922), and Gracanin (1932) threw doubt upon the validity of this concept, since no correlation could be demonstrated between water uptake and salt uptake.

On the contrary the experiments of Haas and Reed (1927), Schmidt (1936), Freeland (1936, 1937), Jahnel (1938), Bötticher and Behling (1939), Wright (1939) and Phillis and Mason (1940) showed clearly the existence of such a correlation.

During the same period the investigations of Steward and coworkers (1936), Hoagland and Broyer (1936), Lundegårdh and Burström (1933), Van den Honert (1933, 1936), Lundegårdh (1940) and Broyer and Hoagland (1943) showed that, in contrast with the water absorption, the salt uptake was a fully active process dependent on the respiration and according to Lundegårdh more particularly dependent on the anion respiration. A passive uptake of ions in the transpiration stream is not consistent with this view. The consequence was that an influence of the water absorption on the salt uptake was thought to be of minor importance if any at all and in the newer textbooks on plant physiology this influence is barely discussed.

Huber (1953) in a recent review supports an intermediate point of view. He assumes that there is an active salt uptake, on which at strong transpiration a passive carrying along in the transpiration stream is superposed. This idea is especially prompted by the results of his pupils Schmidt, Jahnel and more recently by Petritscheck (1953). A definite correlation between water uptake and salt uptake could also be shown by Michael and Wilberg (1951), whereas Hylmö (1953) on account of his experiments with *Pisum sativum* reverted to the conception that the uptake of ions occurs completely passively with the water.

Butler (1953) does not object to this point of view, but shows with his material, *Triticum vulgare*, that the ion transport to the shoot can be inhibited by 2,4-dinitrophenol without any reduction of the water uptake.

In this connection it is worth noting that Long (1943) could reduce the water uptake of tomato plants to about 20 % of the original value without inhibiting the nitrate uptake. The reduction of the water uptake was obtained by the application of an osmotic counter suction in the outer solution.

These methods, inhibition of the salt uptake without reduction of the water absorption and reduction of the water uptake without influencing the salt uptake will give a better insight into the mutual relation between water absorption and salt uptake.

From the facts, mentioned above, it is clear that the different points of view can only be reconciled with each other, by a theory which takes all these experiments into account. It is unlikely that the differing results must be attributed to methodical errors, since both types of results have been corroborated repeatedly. There may be, therefore, an influence of the transpiration on the salt uptake, but on the other hand there need not necessarily be such an influence. Hoagland and Broyer (1936) pointed to the importance of the salt status of the plant in this problem. In this investigation the attention will be drawn to a number of other factors which can be important.

At any rate, it has become apparent that a part of the total salt uptake is active, viz. it takes place at the expense of respiratory energy. This is, why some experiments are performed in order to determine the respiration intensity of the different root zones.

Finally it is of great importance to compare the physiological results with the anatomical structure. We stated above that an interpretation merely on the basis of the anatomical data, may lead us to misinterpretations.

Chapter II

MATERIAL AND GENERAL METHODS

§ 1. Material

In all the experiments described below, 5 to 7 weeks old *Vicia faba* plants were used. After germinating the seeds in wet river sand the seedlings were grown in jars containing tap water. After the removal of the tip of the main root long unbranched secondary roots develop. The length of these secondary roots amounted to 10–22 cm, the diameter was about 1 mm and was nearly constant over the entire length of the root.

§ 2. Determination of the water uptake

The aim of a former investigation was the determination of the water uptake of the different root zones. Besides, it was necessary to measure the suction tension in the xylem vessels of the root according to a method developed by Brewig (1937). For that purpose a rapid change of the outer solution of each zone was also necessary. Both conditions are fulfilled by an apparatus as described in detail in a former paper (Brouwer, 1953*a*). For the sake of brevity we may refer to that paper.

§ 3. Determination of the salt uptake

When determining the water uptake it was possible to change the outer solution after short periods since the experimental periods lasted only 20 or 30 minutes. For the salt uptake such short periods cannot be used, because the uptake of a 3 cm zone of the root is very small in proportion to the sensitivity of the determination methods.

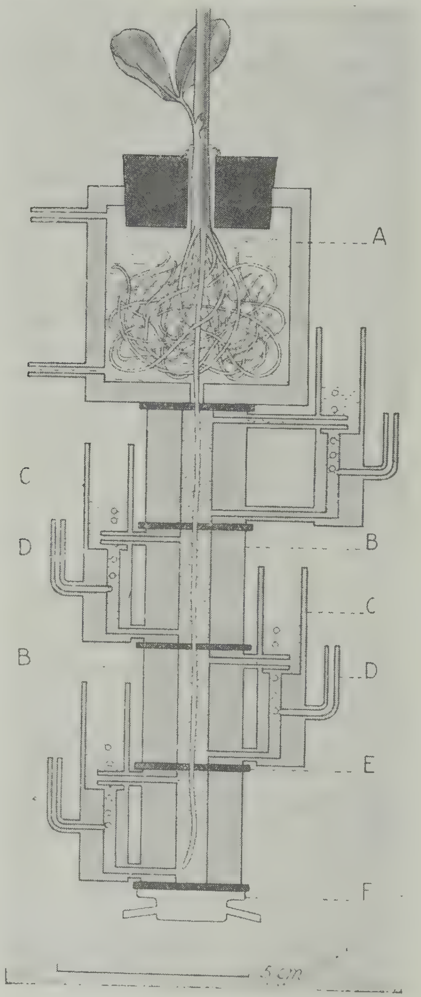

Fig. 1. Schematic longitudinal section of the apparatus used for experiments upon the salt uptake of the different root zones.

A = main vessel, containing the bulk of the roots;
B = zone vessel, containing a single zone of the tested root;
C = aeration vessel connected with B by two transverse tubes;
D = side tube connected with the air container, admitting a constant stream of air bubbles;
E = air- and water-tight rubber disk, fitting around the root, separating two adjacent zone vessels;
F = metal disk; strong springs attached to this disk press the series of zone vessels against the bottom of the main vessel A and hold the apparatus firmly together.

It is necessary, therefore, that the contents of the small vessels be aerated and stirred continually. To satisfy these conditions small vessels were designed which are drawn in section in figure 1. As with the apparatus formerly described for the water uptake, the ground plan is a metal standard on which the main vessel is mounted. Through a hole in the bottom of the main vessel a single root is hanging down. This root is enclosed in a series of small vessels, the zone-vessels.

These zone-vessels are separated from each other by rubber disks with a hole in the centre. Each vessel consists of a central cylindrical block(B) with a cylindrical boring of 8 mm diameter, through which the root hangs. With transverse connections at the top and at the bottom of the vessel it is connected with a second smaller vessel(C) which renders possible a stirring of the contents of vessel B. Via the side tube D a constant air stream enters each vessel C. In this way the solution of the two vessels can be circulated by means of air bubbles, and is aerated and stirred at the same time. Finally there is made a side tube at the bottom of B (not drawn on the figure) by which it is possible to drain off the contents of the vessels quantitatively. For this purpose the side tube is supplied with a rubber hose with a clamp.

The method used is as follows. With a 3 cc pipette a nutrient solution is brought into the vessels. The whole of vessel B and vessel C up to the level drawn on the figure is then filled. After an uptake period the remaining nutrient solution is drained and caught. Thereafter the vessel is rinsed twice with 5 cc distilled water. This is also caught.

To get information with regard to losses during the manipulations and the experiment we have always used one vessel at the bottom of the apparatus, in which no root segment was enclosed. It appeared that in this vessel the original amount of ions could always be recovered completely. With the set up described here the root remained in good condition for a long time.

We have investigated the nitrate, phosphate and chloride uptakes of the various root zones. This took place in separate experiments since the total amount of nutrient solution was always used up by a single determination. The experimental periods lasted from 8 to 24 hours, dependent on the ion investigated. The whole apparatus, with the exception of the metal standard, was made from perspex.

The nitrate analysis took place according to the colorimetric method with phenoldisulphonic acid. The precision amounted to about 3 γ.

The phosphate uptake was determined from solutions with labelled phosphate (P*). The results obtained with the counting method were checked by the colorimetric analysis using the molybdenumblue method. The use of P* made it possible to determine the uptake and the binding in the tissue at the same time.

The chloride analysis was performed by means of the electrometric titration with a 10 mM silver nitrate solution. With some practise the accuracy of this method is about 1 γ Cl. The added silver nitrate was determined gravimetrically.

The uptake periods lasted 24 hours for nitrate, 16 or 24 hours for phosphate and for chloride periods of 8 hours were sufficient.

§ 4. DETERMINATION OF THE OXYGEN UPTAKE

For the determination of oxygen uptake from the medium the apparatus described for water uptake measurement was used. With a syringe pipette, as described by VAN DAM (1935), a sample was taken from each of the potometers separately, sucking off the sample via the lowest side tube (BROUWER, 1953*a*, fig. 1D). The oxygen content of the samples was determined by VAN DAM's adaptation of the micro-Winkler method. By determining this oxygen content before and after an experimental period of 45 or 60 minutes the amount of oxygen withdrawn from the medium could be calculated. The oxygen uptake of all the zones was determined separately at low and high water uptake, both with and without ions being present in the medium.

CHAPTER III

CHANGES IN WATER CONDUCTIVITY OF THE ROOT TISSUE

§ 1. INTRODUCTION

In the literature many experiments are described in which the water uptake by the different zones of the root was determined. At first they were performed by greasing with a fatty substance the whole root with exception of the tested zone, so that the water uptake in the greased zones was impossible. A second method frequently applied was the use of indicators. The roots were allowed to absorb water from a dye solution and after the experiment microscopic tracings showed what parts of the root held the highest concentrations of the dye.

The only thing that was proved by these experiments was the fact that the root was able to absorb water over its whole length. To get more exact data it was necessary to use micropotometers enclosing the different zones of the root.

In 1935 SIERP and BREWIG succeeded in enclosing a single root of *Vicia faba* in a number of potometers, making it possible to determine separately the water uptake of each 2.5 cm zone. ROSENE (1937) described a method for determining the water uptake of very small root segments at different distances from the apex. Moreover, GREGORY and WOODFORD (1939) and HAYWARD, BLAIR and SKALING (1942) described potometer methods. Only the method of SIERP and BREWIG (executed by BREWIG) and the technique of ROSENE appeared useful and following the first descriptions BREWIG and ROSENE published a series of papers, each of them acting with a distinct aspect of the water uptake.

The method used by the author is an improvement of the method of GREGORY and WOODFORD.

As to the results of former potometer investigations only the results of SIERP and BREWIG, BREWIG, and ROSENE need further discussion because the other investigations are more preliminary.

SIERP and BREWIG showed that the water uptake of the different zones of the root strongly varied according to changes in transpiration of the shoot. At low transpiration the maximum water uptake took place near the tip. The basal zones showed a very small water uptake under these circumstances. At high transpiration the water uptake of all zones increased. The water uptake of the basal zones was then higher than the uptake of the apical zones. It is known that according to the formula of BRIEGER (1928) water uptake is determined by the suction tension in the xylem vessels and the water conductivity of the root tissue between medium and vessels: Uptake = c × S.

BREWIG (1937) showed that the suction tension in the xylem vessels in the apical parts is as high as in the basal zones. The conductivity for water, therefore, is highest in that region, where the water uptake is highest. Hence it is clear that at low transpiration the conductivity is highest in the apical zone and small in the basal ones. At high transpiration the conductivity of the apical zone remains the same as before but the conductivity of the basal zones shows an important increase. The sole assumption that we have to make is that the pathway of the water is the same at low and high transpiration. It might be conceivable that at high suction channels open up which are not available at low transpiration, for instance a lengthwise transport through the cortex. With regard to such a transport two possibilities can be considered. Firstly a transport through the walls is possible. In view of the narrow connecting canals (intermicellar spaces) and the length of the pathway such a transport would constitute an almost unfeasible task for the plant. As to an osmotic transport from cell to cell the same objection holds. Therefore, BREWIG is justified in stating that the resistance to water transport is localised in the tissue between medium and vessels.

The phenomenon that is at issue is the change in resistance against the water current, particularly in the basal zones. The changes in water conductivity are correlated with increases in suction tension. BREWIG (1939) even succeeded in getting the same changes in conductivity with excised roots by applying an osmotic suction (strong sucrose solution) in the medium, but only if at the same time, the contents of the xylem vessels were continually sucked away. By applying this sugar solution in the medium, water was withdrawn from the root segment as long as a water current was maintained lengthwise through the xylem vessels. As soon as this water stream stopped, the withdrawal by the sugar solution decreased rapidly. WIERSUM (1944) who showed with the same material, that sugars could diffuse rather easily through the tissue from medium to xylem vessels and vice versa, concluded from BREWIG's result that with a stagnating water stream in the xylem vessels sugar diffusion from the medium to those vessels would be responsible for a decreasing difference between the osmotic value of the contents of the vessels and the medium. This would result in the decreasing withdrawal of water by the sugar solution.

ROSENE (1941) working with both excised and intact onion roots, found that the water uptake was considerably increased following a treatment with sucrose in the medium. It seems that the treatment with sugar solution makes the tissue more permeable to water. Although this interpretation is not considered by the authors mentioned, it seems wise to bear in mind this possibility, in view of the experiments described below. The problem that is at issue, therefore is in the first place the causation of the changes in water conductivity.

Besides, it seems important to get some information regarding the area where these changes in conductivity take place; in the protoplasm, in the wall or in both. In the beginning BREWIG assumed the protoplasm to play an important role in these changes and he gives calculations from which it appears that the permeability of the protoplasm of the root of *Vicia faba* was of the same order as the permeability calculated by HUBER and HÖFLER (1930) for Salvinia and other objects. A transport through the protoplasm was rejected by him, however, after noting the results of STRUGGER (1938). STRUGGER showed by means of fluorescing dyes as indicators a wall transport of water in parenchymatous tissues. It should be remembered that the endodermis will raise a barrier for a transport through the walls.

§ 2. THE CAUSAL RELATIONS OF THE CHANGES IN WATER CONDUCTIVITY

If we look upon the distribution of water uptake over the different zones of the root, it appears that at low transpiration the maximum amount of water is taken up by either the apical zone or the zone adjacent to it. If the external circumstances are not altered a steady state is obtained which shows only slight fluctuations.

Enhancing the transpiration produces an immediate increase in uptake in such a way that the ratio between the rate of absorption, before and after enhancement, is the same for all zones. This must be due to the increased suction tension in the xylem vessels. For, according to the formula of BRIEGER (Uptake = Conductivity × Suction tension) the water uptake is determined by the suction tension in the xylem vessels and the water conductivity in the root tissue. This applies to the root system as a whole as well as to each zone separately. If the suction tension increases to twice its original value, the water uptake ought to increase to twice its original value, assuming the conductivity to be constant. After some time, however, we see that the water uptake of the more basal zones becomes greater and greater. After about three hours a new steady state has been established and a new constant distribution of the water absorption rates between the different zones has been reached. The difference with the former distribution (at low transpiration) is that the maximum uptake is shifted to the basal zones. As compared with the situation at low transpiration there is a difference in two respects. Firstly the suction tension is increased but this increase is the same for all zones. As a consequence of this fact therefore, the distribution of the uptake over the root axis would not change. On the other hand the conductivity is changed and these changes in conductivity caused the shifting of the uptake maximum.

It appears that the conductivity of the apical zones has not changed at all, whereas the conductivity of the basal zones is strongly increased. In this case, therefore, the changes in conductivity were produced by an increase of the suction tension in the xylem vessels as a consequence of an increased transpiration. However, an increased suction tension in the vessels can be produced in another way.

With the aid of the set up described it is easy to enhance the water absorption of the tested root by applying an osmotically active solution to the bulk of the roots in the main vessel. The water uptake of the plant as a whole is more or less hampered by this manipulation. In this way it is possible to get every desired suction tension in the xylem vessels and to investigate the correlation between suction tension and conductivity.

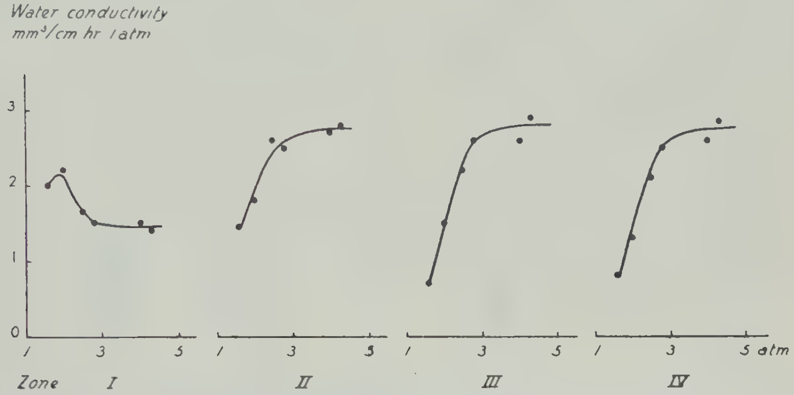

Fig. 2. Relationship between water conductivity of the various root zones and suction in the vessels. This relation has been plotted for each zone separately. The increase of the suction tension in the xylem vessels from 1.6 atm at the start of the experiment to 4.3 atm at the end was effected in the following ways. Exposing the plant to light resulted in an increase from 1.6 to 2.0 atm. Further increases were obtained by applying a higher salt concentration to the roots in the main vessel; with 1.0 atm Brenner solution the suction tension increased from 2.0 to 2.5 atm; with 1.5 atm Brenner solution from 2.5 to 2.8 atm; with 3.3 atm Brenner solution from 2.8 to 4.0 atm; and with a 3.9 atm Brenner solution from 4.0 to 4.3 atm. The conductivity values at a suction tension of 1.6 atm show that the normal distribution at low suction tension occurred at the start of the experiment. All the values plotted are determined at a steady state, the water uptake and the suction tension being constant for several hours.

The results of such an experiment are plotted in figure 2 (ample description of the experiment vide BROUWER 1953*a*). In this figure the conductivity of the different zones is plotted against values of the suction tension of 1.6; 2.0; 2.5; 2.8; 4.0 and 4.3 atmospheres. It is clear that the water conductivity of the basal zones increases from about 0.6 mm³/cm.hr. 1 atm at 1.6 atm to about 3 mm³/cm.hr. 1 atm at 4.0 atm. The conductivity of zone II is already comparatively great at low suction tension. In spite of this it further increases with increasing the suction tension. The conductivity of the tip zone (zone I) initially increases a little but thereafter decreases.

We have to consider two possibilities for this increase in water conductivity. It is possible either that the existing channels increase their capacity for water transport or that new channels, also running in radial direction, are added to the existing pathway. It is difficult to make a choice between these two possibilities on the basis of these experiments. It is clear, however, that there exists a strong correlation between the suction tension in the xylem vessels and the water conductivity of the root tissue.

On the basis of the result of ROSENE (1941) we may expect that the same correlation exists between suction tension in the outer solution and water conductivity of the root tissue. To check this assumption we carried out the following experiment; the results are plotted in figure 3.

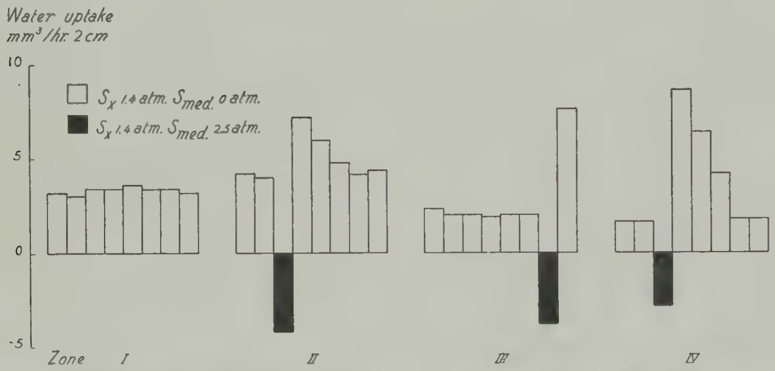

Fig. 3. Comparison of the water uptake of the different root zones, before, during and after application of a 2.5 atm sucrose solution. Each block represents the uptake value during one hour period in mm per hour per zone of 25 mm length. The suction tension in the xylem vessels amounted to 1.4 atm and remained constant throughout the experiment. Applying the 2.5 atm sucrose solution (black blocks) resulted in a withdrawal of water from the root. After this application the amount of water taken up was considerably greater than before. The ratio between the value before and after the application of sucrose is a measure of the changes in conductivity, the suction tension in the xylem vessels remaining the same in both cases.

The water uptake of three zones of the tested root of a plant with low transpiration and a low suction tension in the xylem vessels (1.4 atm) is measured before, during and after the application of a 2.5 atm sucrose solution in the medium. The tip zone is taken as control. The distribution of the water uptake over the different root zones at the start of the experiment was the normal one at low suction tension; the water uptake being highest in the apical region (zone I and II). During application of the sugar solution a withdrawal of water took place, the osmotic suction tension of the sugar solution being higher than the suction tension in the xylem vessels. The sugar solution is applied during the third one hour period to zones II and IV and during the seventh one hour period to zone III. During the sugar application the water moving power amounted to 2.5 — 1.4 =

1.1 atm. During the other periods this power amounted to 1.4 atm. For calculating the water conductivity during the successive periods we have to divide the water uptake by the effective suction tension. In table I the water conductivity values of the different root zones are given of the period directly before, during and directly after the sugar application. It appears from this table that the conductivity during a period of sugar application (one hour) is greater than in the preceding period. During the period directly after the sugar application the water conductivity is much higher than during the period before the treatment with sugar. It seems that because of the sugar application the conductivity increases and that this increase needs some time to get its highest value. The ratio between the conductivity values before and after the sugar treatment amounts to about 5.0 for zone IV, to about 4.0 for zone III and to about 1.6 for zone II. This means that the changes in water conductivity as caused by treatment with a sugar solution are most important in the basal zone. It is striking, therefore, that this behaviour resembles greatly the changes in conductivity obtained by an increase in suction tension in the xylem vessels.

TABLE I

The water conductivity of three zones of the tested root, directly before (*a*), during (*b*) and directly after (*c*) application of a 2.5 atm sucrose solution in the medium (vide fig. 3). The suction tension in the xylem vessels amounted to 1.4 atm.

Zone		Water transport mm³/cm. hr	Effective s. t. atm	Water conductivity mm³/cm. hr. 1 atm
IV	*a*	0.8	1.4	0.6
	b	1.4	1.1	1.3
	c	4.3	1.4	3.1
III	*a*	1.0	1.4	0.7
	b	1.9	1.1	1.7
	c	3.8	1.4	2.7
II	*a*	2.0	1.4	1.5
	b	2.1	1.1	1.9
	c	3.6	1.4	2.6

After some time the conductivity decreases to its original value. This is obvious from the steadily decreasing water uptake values after sugar application.

It is possible to get these changes in water conductivity at constant suction tension in the vessels in different ways. Instead of a sugar solution an osmotically active salt solution (Brenner solution) can be used. Moreover, it is possible to enhance the water conductivity of a root zone by means of a slight drying up of the tissue, e.g. by an air current through the potometer for 3 or 5 minutes.

All these methods have the same effect, namely, a water withdrawal from the tissue and a reduction of the turgescence. This holds for an increased suction tension in the xylem vessels caused by a loss of water during transpiration, as well as for an osmotic suction tension applied in the medium.

§ 3. LOCALISATION OF CHANGES IN WATER CONDUCTIVITY IN THE PROTOPLASM

To investigate the localisation of the resistance to the water current we have to look for the factors which influence the conductivity. RENNER (1929) showed already that the conductivity of a whole root system could be enhanced by killing the root tissue by means of high temperatures. From these results he concluded that the living root tissue and especially the living protoplasm, offered considerable resistance to the water current. KRAMER corroborated this observation and got the same result with ether narcosis. The cold-hardening experiments of LEVITT and SCARTH (1936) and the carbon-dioxide influence of CHANG and LOOMIS (1945) and of HOAGLAND and BROYER (1942) point in the same direction.

ROSENE (1944, 1947, 1950) described investigations into the influence of respiratory inhibitors and oxygen deficiency on the water uptake and exudation of excised onion roots. She found a strong inhibition at definite concentrations. It seems possible, however, that the salt secretion was inhibited in these experiments and that this inhibition caused a decreased water absorption. VAN ANDEL (1953) showed that even in a medium of distilled water a salt secretion into the xylem vessels is possible (tissue bleeding). BROUWER (1953*a*) found that the effects of a number of factors locally applied remained confined to the treated zones, so that without influencing the total water supply of the plant, the effects of several factors on the conductivity of the root tissue of the treated zone could be investigated.

By narcosis with chloroform the conductivity increases, confirming the ether experiment of KRAMER. Applying a carbon dioxide saturated solution reduced the conductivity (confirming the results of CHANG and LOOMIS and of HOAGLAND and BROYER), while, as KRAMER found, oxygen deficiency had little influence. Only the tip zone showed a gradual decrease in water uptake in the latter case.

All these experiments fail to detect an active water uptake during high transpiration. This also appeared from the influence of potassium cyanide on the water uptake. Concentrations of 10^{-4} and 10^{-5} molar, which inhibit respiration, do not influence the water uptake. Very high concentrations of 10^{-3} and $5.\ 10^{-3}$ molar have a distinct influence.

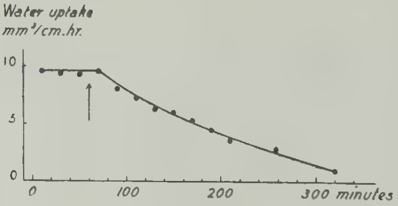

Fig. 4. Time course of the influence of potassium cyanide on the water uptake by one zone of a single root of an intact plant. The suction tension in the xylem vessels and the water uptake of the adjacent zones (non-treated) remained constant. More than 4 hours elapsed before the uptake had been diminished to about 10 % of its original value. The arrow indicates the beginning of the treatment.

Applying 5×10^{-3} M KCN stops the water uptake immediately. The time course of the influence of 10^{-3} M KCN on the water uptake is plotted in figure 4. The experimental plan was the following. At high water uptake, in the outer solution of a basal zone the tap water was replaced by a 10^{-3} M KCN solution. A gradual reduction of the water uptake was the result of this treatment. The water uptake of the adjacent non-treated zones remained unchanged, indicating that the suction tension in the xylem vessels did not alter. The water uptake of the treated zone was reduced gradually until after about 5 hours it completely stopped. Not until 36 hours after removing the potassium cyanide was this uptake normal again. It is unlikely that this gradual effect is attributable to an elimination of an active component of the water uptake. Rather we are dealing with a change in the structure of the protoplasm slowly setting in, causing a greater resistance to the water current. From these experiments we have to conclude that water conductivity is determined by the protoplasm to a high degree.

§ 4. DISCUSSION

The experimental results show that by applying an osmotic suction tension in the medium the conductivity for water in the root tissue is increased. This increase is identical with that caused by normal changes of the suction tension in the xylem vessels. This demonstrates that the changes in conductivity are dependent on changes in suction tension.

Moreover, it is clear that this is not a polar process, since an enhancement of the suction tension in the xylem vessels has the same effect as an increase of the suction tension in the medium. In both cases the suction tension of the cells of the root tissue is influenced, the turgidity being reduced. It may be assumed, that this decrease in turgidity is responsible for the increase in conductivity.

Further, the experiments with inhibitors, oxygen deficiency and carbon dioxide treatment point to a plasmatic process, that changes the water conductivity of the tissue. Thus we may conclude that somewhere between the medium and the xylem vessels migration of water takes place through the cytoplasm. The exact area where this takes place is still unknown. Increasing the suction tension of the cell reduces the turgor pressure acting upon the protoplasm between vacuole and cell wall. It is supposed that this decrease in pressure upon the protoplasm causes a greater water conductivity of the protoplasm. The inhibitors etc. are assumed to be active via the structure of the protoplasm.

It is not necessary to suppose that there is any active water uptake.

It may be assumed that the same influence of an osmotic suction tension in the medium on the water uptake after treatment was found earlier but was not interpreted in this way.

Chapter IV

THE ANION UPTAKE OF THE DIFFERENT ROOT ZONES UNDER VARIOUS CIRCUMSTANCES

§ 1. Introduction

As stated in chapter I, up to now, little has been known about the salt uptake of the different zones of the root. The experiments of Gregory and Woodford are preliminary and general conclusions are not yet possible. After developing a method for the determination of water uptake by the different zones, Brewig discussed the desirability of a determination of the salt uptake. He was not successful in realizing his idea. It is clear that many physiologists before and after Brewig have seen the desirability of such experiments. It is probable that technical difficulties have been responsible for the lack of such like experiments. The fact that the secondary roots of *Vicia faba* are strong enough and reach a length of about 20 cm before branching takes place, makes this material suitable for potometer experiments. Further, the possibilities offered by modern substances like perspex have contributed greatly to the mastery of the constructive difficulties.

As stated in chapter II a root of an intact *Vicia faba* plant is enclosed in a series of small vessels, each enclosing 3 cm of the root, and each of which can be easily drained. Joining an aeration mechanism to these vessels makes it possible to perform rather prolonged experiments. The plant was illuminated by a 300 Watt lamp at a distance of 40 cm and placed in a chamber of constant temperature, 24° C ± 0.5° with a relative humidity of 40–45 %.

In spite of these constant circumstances, it was possible to vary the water uptake of the root in the zone-vessels. This could be achieved by changing the osmotic suction tension of the solution in the main vessel in which the bulk of the root system was placed. As stated above this renders it possible to get each value of the suction tension in the xylem vessels and corresponding values for water uptake by the tested root. A salt solution according to Brenner (1920) of different concentrations was used as an osmotically active solution. Because this solution contains much chloride a sucrose solution was used during experiments in which the chloride uptake was determined. A mannitol solution which has often been applied for this purpose, was not suitable in this case for it proved to reduce the water uptake very little.

The experiments were performed as follows. After enclosing the root in the zone vessels the main vessel was filled with tap water and the zone vessels were filled with 3 cc of the nutrient solution. After about 4 hours the contents of the zone vessels were drained and the vessels rinsed twice with 5 cc distilled water. Thereafter each zone vessel was refilled with 3 cc of the nutrient solution and the experiment was started. The preceding period of 4 hours was taken to allow an exchange equilibrium between the root and the nutrient solution to be established. After an uptake period of 24 hours (in a few cases

8, 12 or 16 hours) the nutrient solution was tapped off from the zone vessels and the vessels were rinsed twice with distilled water. This rinsing water was caught and mixed with the former sample. Apart from the number of vessels enclosing the root, there was always one vessel that served as a control.

The quantity of ions investigated was determined in:

a. 3 cc of the original nutrient solution;
b. the control after 24 hours aeration, rinsing etc.;
c. each of the zone-vessels after a 24 hour uptake period.

It appeared that the amounts found in *a* and *b* were the same within the limits of analytical errors.

This means that there are no losses as a consequence of the various manipulations. The difference between *b* and each of the zone vessels was taken as the uptake of the root zone concerned. All values were calculated in γ per 24 hours per 3 cm root length.

In previous experiments the constancy of the uptake under constant circumstances in successive periods of 24 hours had been investigated. It appeared that the uptake of the different zones was constant at least during three successive periods of 24 hours. This was the case with low water uptake as well as with high water uptake. This means that it is possible to investigate the influence of various factors on the ion uptake within a time period of 72 hours. The experiments described below, therefore, lasted 72 hours at the most.

§ 2. INFLUENCE OF WATER UPTAKE ON ANION UPTAKE BY THE DIFFERENT ZONES OF THE ROOT

The experimental planning of all these experiments was the following. The uptake of the nitrate, chloride or phosphate ions was determined during two successive periods of the same duration. During one of these periods there was tap water in the main vessel, during the other a Brenner salt solution or a sucrose solution of about 2.5 atm. This means, therefore, that on the ground of preceding experimental results the water uptake by the tested root can be assumed to be low with a maximum near the tip when tap water is in the main vessel and to be high with a maximum in the basal zones when an osmotically active solution is in the main vessel. The shoot remained under constant conditions during both periods. The anion uptake of the two periods was compared.

a. Chloride uptake

In connection with the fact that the analysis of chloride by means of the electrometric titration with silver nitrate makes possible very accurate determinations most of the experiments were performed with chloride. The chloride uptake from a 5 mM calcium chloride solution was determined. The chloride uptake of the different root zones at low and high water uptake is recorded in table II. From these data we see that the chloride uptake at high water uptake is considerably greater than at low water uptake.

TABLE II

Chloride uptake of the different root zones at low and high water uptake in γ Cl per 24 hours per 3 cm root length. Average values for 5 experiments

Zone	Water uptake by the tested root		Ratio
	low	high	
IV	12 γ Cl	42 γ Cl	3.5
III	19 ,,	52 ,,	2.7
II	26 ,,	43 ,,	1.7
I	26 ,,	33 ,,	1.4

At low water absorption the chloride uptake is highest in the apical zones and diminishes towards the base. At the transition from low water uptake to high water uptake the chloride uptake of the apical zones increases least. The greater the distance from the apex the greater the percentage increase in uptake. This agrees with the reaction which we found in the experiments on water uptake. There is, however, a quantitative difference. The maximum water uptake at a high suction tension in the xylem vessels was always found in the basal zones. This is not the case here. The relative increase in water uptake was greater than the increase in chloride uptake found here.

b. *Nitrate uptake*

In this case the difficulty arose that the nitrate uptake of the basal zones hardly exceeded the errors of the analysis when the water uptake was low. When water absorption was high, however, a significant nitrate uptake was obtained. To get a better view of the quantitative relations of this uptake at low and high water uptake the average values of this uptake in 8 experiments were determined. In table III all results are given and in a separate column the average values at low and high water uptake are shown. The corresponding experiments are indicated by experimental numbers one to eight. The ratio is calculated from the average values.

TABLE III

Nitrate uptake of the different root zones at low and high water uptake in γ N per 24 hours per 3 cm root length

Zone	Water uptake by the tested root																		Ratio
	low									high									
	Exp. nr.								Av. γN	Exp. nr.								Av. γN	
	1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8		
IV	8	4	2	9	3	5	–3	8	4.5	26	23	8	19	16	17	13	27	18.6	4.1
III	12	6	4	16	4	7	–2	10	7.1	30	26	8	20	15	19	12	25	18.4	2.6
II	11	8	6	19	9	8	4	10	9.4	28	24	16	23	18	23	10	29	21.4	2.3
I	13	12	6	18	12	10	7	12	11.6	19	17	10	24	17	16	14	18	16.9	1.4

The nutrient solution used was a 10 mM potassium nitrate solution. From the data of table III it appears that for nitrate the same holds good as for chloride, i.e. with an increased water uptake an increase of the nitrate uptake takes place simultaneously. This increase is also highest in the basal root parts.

c. Phosphate uptake

The phosphate uptake is determined with the aid of radio-active phosphorus. A full strength Hoagland solution containing labelled phosphate (activity about $15\mu^{\mathrm{Curie}}/_{\mathrm{L}}$) was used in the zone vessels. The activity before and after an uptake period was determined. The possibility exists that there has occurred some exchange of radio active phosphate against phosphate already present in the root tissue. This exchange, however, is probably negligible, for radio-active phosphate taken up by the root did not show any exchange with unlabelled phosphate in the medium. As is shown in table IV the phosphate uptake gives essentally the same picture as the chloride and nitrate uptake. The data reported refer to a single experiment. The root used for this experiment was 15 cm long. Zone I showed a rapid growth. The uptake of this zone (per 3 cm root length) was about the same under the various circumstances. The mature zones III and IV demonstrated the greatest influence of the water uptake on the phosphate uptake. The root segment V displayed an incipient side root formation during the experiment. We see that the phosphate uptake of this zone at low water uptake was higher than the uptake of the adjacent zone IV. On the other hand the influence of the water uptake was smaller in zone V. Thus the nature of the uptake resembles that of the apical zones. As will be described in one of the following chapters this is due to a relatively greater binding of phosphorus in the root tissue itself.

TABLE IV

Phosphate uptake of the different root zones at low and high water uptake in γ P per 24 hours per 3 cm root length

Zone	Water uptake by the tested root		Ratio
	low	high	
V	4.1 γ P	10.8 γ P	2.6
IV	1.5 ,,	9.9 ,,	6.6
III	1.6 ,,	10.4 ,,	6.5
II	5.2 ,,	9.3 ,,	1.8
I	10.1 ,,	11.3 ,,	1.1

It may be concluded from these experiments that the increase in water uptake is accompanied by an increased anion uptake. These results agree with experiments in which the uptake by the whole root system was determined at low and high transpiration (BROUWER, 1953*b*).

It is clearly demonstrated that this increase in anion uptake is in the main the consequence of an increase in uptake by the basal zones. The question arises how far this increased uptake at high water uptake is an active or a passive process.

§ 3. HAMPERING THE WATER UPTAKE DOES NOT INFLUENCE THE INCREASED ANION UPTAKE

These experiments were started in the same way as those of the preceding paragraph, viz. the first period with tap water in the main vessel, the second period with a 2.5 atm sucrose solution. In this series the chloride uptake from a 5 mM calcium chloride solution was determined for both periods. During the third period conditions in the main vessel remained as in the second period. The different root zones could then absorb from a 5 mM calcium chloride solution to which sucrose was added until an osmotic value of 2.5 atm was reached. The water uptake of the enclosed root zones may be assumed to be reduced compared with that uptake during the second period. No reduction of the chloride uptake can be detected, however. All the experiments performed in this way with sucrose as an osmotic substance showed this result.

The results of a single experiment are plotted in figure 5. From this figure we see that increasing the water uptake of the tested root (transition from period 1 to 2) is followed by an increase in chloride uptake. Reduction of this water uptake by a counter suction in the medium (period 3) does not reduce the increased chloride uptake, on the contrary this uptake continues to take place at the preceding high level.

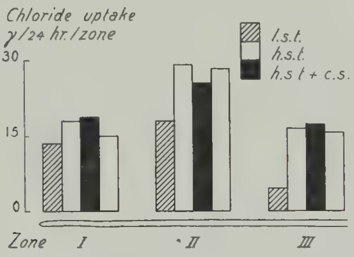

Fig. 5. The chloride uptake of the various root zones; shaded blocks, low suction tension in the vessels; white blocks, high suction tension in the vessels; black blocks, high suction tension in the vessels with an osmotic counter suction in the medium. The outer solution was 5 mM $CaCl_2$ (period 1,2 and 4) and 5 mM $CaCl_2$ + 100mM sucrose (period 3). Each period lasted 24 hours. The shoot was constantly illuminated. The suction tension in the vessels of the tested root was increased by enhancing the osmotic value of the solution in the main vessel.

We suppose, therefore, that the anions are not carried along passively with the increased transpiration stream during period 2, for in that case we would expect that the anion uptake, as a result of the reduction of the water uptake by the sugar solution in the zone vessels, would also decrease.

These experiments were repeated with mannitol as an osmotic agent. The results obtained were more variable. From parallel experiments upon the influence of mannitol on water uptake it appeared that this substance either inhibited water uptake to a variable extent or failed to do so. In some of the experiments with mannitol on the chloride uptake a leakage of chloride ions could be demonstrated. This leakage sometimes continued during a following period after the removal of the mannitol. An explanation of this phenomenon can not be given as yet.

§ 4. Inhibition of anion uptake at low and high water uptake

From the preceding experiments we may conclude that the anion uptake increases simultaneously with water absorption, but that the anion uptake is not directly dependent on water uptake since it is possible to reduce the latter without influencing the anion uptake.

It was expected that by means of inhibitors the reverse could be reached. The experiments were performed in the same way as described above. The inhibitors used were applied in concentrations which did not influence the water uptake according to former experiments.

The experiments were carried out in order to be able to decide whether the increased anion uptake at high water uptake was an active or a passive process. In the former case it must be possible to inhibit a greater portion of the total anion uptake than the amount of ions already taken up at low water uptake. From table V it is clear that the inhibition (difference between columns 2 and 3), recorded there, is slightly higher than the uptake values at low water uptake. Thus, although these data do not prove definitely that the inhibition is greater than the uptake at low suction tension, this seems very likely.

TABLE V

Chloride uptake of the different root zones in γ Cl per 24 hours per 3 cm root length at low and at high water uptake. Column 3 shows Cl uptake at high water uptake and with 10^{-5} M DNP added to the nutrient solution

Zone	Water uptake by the tested root		
	low	high	high + 10^{-5}M DNP
IV	7 γ Cl	22 γ Cl	12 γ Cl
III	11 ,,	27 ,,	14 ,,
II	13 ,,	24 ,,	9 ,,
I	12 ,,	19 ,,	6 ,,

When higher concentrations of the inhibitor are used it is possible to completely inhibit the chloride uptake. The drawback of such experiments is, however, that sometimes a leakage from the root can be measured which renders the interpretation more suspicious.

Investigating the inhibition by definite concentrations of the inhibitors at low and high water uptake it was apparent that different plants showed a great difference in sensitivity for inhibitors. Moreover, in no case did this inhibition appear to be fully reversible, i.e. the anion uptake before and after application of the inhibitor did not reach the same level. This rendered it impossible to investigate the inhibition at low and high water uptake with the same root. The consequence is that we are obliged to derive our conclusions from a comparison of different experiments, accepting the variability of the sensivity of the individual plants. We traced the effect of potassium cyanide 5 × 10^{-6}, 2–4-dinitrophenol 10^{-5}, 0_2-deficiency by nitrogen bubbling through the solution, 0_2-deficiency by stopping the aeration, and CO_2 bubbling through the solution.

The results of inhibition of the chloride uptake by 10^{-5} M 2,4-dinitrophenol (DNP), 5 10^{-6}M potassium cyanide and nitrogen are given in table VI, viz. at low water uptake in VI*a* and at high water uptake in VI*b*. From these data it can be concluded that the percentages of inhibiton differ somewhat. The inhibition is always strongest in the apical zone. We can state that we cannot observe any essential difference between the experiments at low water uptake and those at high water uptake. It is a pity that the best method, the direct comparison of the inhibition at low and high water uptake with the same root, cannot be used. This is possible, however, with the inhibition of the chloride uptake obtained by stopping the aeration, such inhibition being fully reversible.

TABLE VI

Inhibition of the chloride uptake by various inhibitors at low and at high water uptake of the tested root

A. Chloride uptake of the different root zones at low water uptake with and without inhibitor

Zone	DNP 10^{-5} M			KCN 5.10^{-6} M			N_2		
	—	+	% inh.	—	+	% inh.	—	+	% inh.
IV	7	4	43				10	6	40
III	16	4	75	5	2	60	11	7	36
II	15	0	100	29	11	63	13	6	52
I	27	6	78	27	6	78	20	7	66

B. Chloride uptake of the different root zones at high water uptake with and without inhibitor

Zone	DNP 10^{-5} M			KCN 5.10^{-6} M			N_2		
	—	+	% inh.	—	+	% inh.	—	+	% inh.
IV	41	25	40	49	30	40			
III	39	26	35	53	28	47	57	23	40
II	49	32	37	50	26	48	40	18	55
I	42	19	55	36	11	70	31	12	61

The data of table VII may illustrate this fact. Comparing the values of columns 1 and 3, on the one hand, and columns 5 and 7 on the other, we see that the uptake after a period without aeration reaches about the same level as that during the period preceding the treatment. Moreover, it appears that the inhibition obtained by stopping the aeration is equally strong both at low and high water uptake.

This strongly supports the fact that the chloride uptake at high water uptake is as active as the uptake at low water uptake.

TABLE VII

Chloride uptake of the different root zones in γ Cl per 24 hours per 3 cm root length with and without aeration at low and high water uptake

Water uptake	low				high			
Aeration	on	off	on	% inh.	on	off	on	% inh.
Zone								
III.	23	19	21	14	58	33	64	46
II.	33	16	35	53	40	18	38	50
I.	31	13	29	57	32	12	26	59

§ 5. DISCUSSION

The experiments on the determination of the anion uptake by the different root zones, described in this chapter, are new with the exception of a few preliminary communications. It is clear that at low water uptake, the anion uptake is strongest within a distance of 5 cm from the tip. On the other hand it is clear that under these circumstances the more basal zones, up to at least 15 cm from the apex are able to absorb anions. This is not surprising since we know that water can be absorbed over the whole root length.

BROUWER (1953*b*) demonstrated that the material used, showed an increased anion uptake after increasing the water uptake. It could be expected, therefore, that one single root would give the same phenomenon as the whole root system. It is interesting that especially the basal zones strongly increase their anion uptake just as they did for water. The anion uptake of the tip zone showed hardly any change. This fact is particularly interesting for we know that the water uptake of the tip zone increased almost in proportion with the increase in suction tension in the xylem vessels, according to the fact that the water conductivity of the tip zone remains about the same. This suggests a probable mechanism as to the influence of the transpiration on the anion uptake.

If the extra amount of ions taken up is assumed to be transported passively by the water stream the tip zone would show an increased anion uptake simultaneously with an increased water uptake. A passive transport in the transpiration stream is the more improbable because we are able to hamper the water uptake by applying an osmotic counter suction in the outer solution without any reduction in anion uptake.

The experiments with inhibitors support this assumption, although the results were variable.

One drawback of the experimental method described in this chapter is the fact that it was impossible to compare directly the water uptake with the anion uptake.

CHAPTER V

DIRECT COMPARISON OF WATER UPTAKE AND ANION UPTAKE IN THE SAME ROOT

§ 1. INTRODUCTION

The drawback of the experiments described in the preceding chapter, viz. the impossibility of getting direct information on the water uptake, made a direct comparison of both water and salt uptake values desirable. The experiments described below were performed in such a way, that the water uptake of two zones of the tested root was determined, simultaneously with a determination of the anion uptake of the other zones. The water uptake was determined during two periods of one hour, one at the start and one at the end of each experimental period. Between these periods a continuous flow of aerated nutrient solution was circulated through the potometers used for measuring the water uptake. In this way conditions in the water uptake vessels and the salt uptake vessels were maintained as equal as possible.

§ 2. SIMULTANEOUS DETERMINATION OF THE INCREASE IN CHLORIDE UPTAKE AND THE INCREASE IN WATER UPTAKE ON ENHANCING THE SUCTION TENSION IN THE XYLEM VESSELS

The suction tension in the xylem vessels was varied in the usual way, viz. by changing the osmotic value of the solution in the main vessel. Tapwater and a 2.5 atm. sucrose solution were used alternately. The uptake values during a period with low suction tension (l.s.t.) and a period with high suction tension (h.s.t.) in the xylem vessels were compared. The results of two experiments are given in table VIII. Table VIIIA gives the data of an experiment in which the chloride uptake of zones I and III and the water uptake of zones II and IV were determined. On the contrary, table VIIIB indicates the water uptake of zones I, III and V, and the chloride uptake of zones II and IV. Though not completely comparable (the experiments

TABLE VIII

Increase in chloride and water uptake of the various root zones as caused by an increased suction tension in the vessels. Chloride uptake in γ Cl, water uptake in mm^3 per 24 hours per zone

Experiment A

Zone	L. s. t.	H. s. t.
$\mathrm{IV}_{\mathrm{water}}$	56	352
$\mathrm{III}_{\mathrm{chloride}}$	7	25
$\mathrm{II}_{\mathrm{water}}$	112	320
$\mathrm{I}_{\mathrm{chloride}}$	32	34

Experiment B

Zone	L. s. t.	H. s. t.
$\mathrm{V}_{\mathrm{water}}$	64	704
$\mathrm{IV}_{\mathrm{chloride}}$	18	37
$\mathrm{III}_{\mathrm{water}}$	84	672
$\mathrm{II}_{\mathrm{chloride}}$	25	35
$\mathrm{I}_{\mathrm{water}}$	152	278

were performed with different roots) the data give a good picture of the influence of an increased suction tension in the xylem vessels on the water and anion uptake of the different root zones. It is, in a sense, a combination of an experiment on the influence of an increased suction tension on the water uptake and an experiment on such an influence on the anion uptake. It is not surprising that the results reported in table VIII confirm the results described above. It is possible to derive from this table a number of important data.

At first it appears that the increase in water uptake is greater than the increase in chloride uptake. This holds good for both experiments.

The chloride uptake of the tip zone does not increase at all although the water uptake does. This is reasonable since the suction tension is enhanced. The increase in water uptake of the tip zone is more or less a measure of the increase in suction tension in the xylem vessels.

The more basal zones show a greater increase in water uptake partly caused by an enhancement of the suction tension and partly by an increase in water conductivity.

The increase in chloride uptake of the basal zones is lesser than the increase in water uptake of these zones.

§ 3. The influence of an osmotic counter suction on water uptake and chloride uptake

These experiments were performed in the same manner as those described in § 3 of the preceding chapter. During the first period the water and chloride uptake from a 5 mM calcium chloride solution was determined at low suction tension in the xylem vessels. In the second period the only difference with regards to period 1 was the enhanced suction tension in the xylem vessels. During the third period the water and chloride uptake from a 5 mM calcium chloride solution

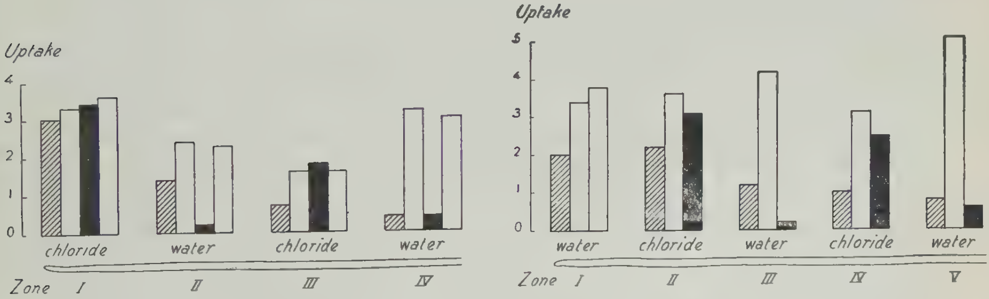

Fig. 6*a*. and *b*. Comparison of chloride uptake and water uptake of the various root zones, at low suction tension (shaded blocks), at high suction tension without a counter suction in the medium (white blocks) and at high suction tension with an osmotic counter suction in the medium (black blocks). Solutions used as indicated in fig. 5. *a* and *b* represent different experiments; *a* with the tip zone enclosed in a salt uptake vessel, *b* with the tip zone in a potometer. With a counter suction in the medium the water uptake strongly decreases without a reduction in chloride uptake. Scale unity = 14 γ Cl or 200 mm^3 water per zone per 24 hr. Experimental periods 16 hours.

with added sucrose (2.5 atm) was determined at a high suction tension in the xylem vessels.

We see from figures 6*a* and 6*b*, showing respectively chloride uptake of the tip zone and water uptake of the tip zone, that on increasing the suction tension in the xylem vessels the normal enhancement of the water and chloride uptake takes place and that during the third period, when an osmotic suction tension in the medium was applied, the water uptake is strongly reduced, without any decrease of the chloride uptake. We have, therefore, a distinct affirmation of the supposition stated in the preceding chapter.

In figure 7 the results are plotted of an experiment in which the counter suction in the medium was applied at low suction tension in the xylem vessels. The osmotic value of these solution was 2.5 atm, as in the former experiments. After applying this solution we see an immediate water withdrawal from the root. This withdrawal is diminishing gradually and reverted into a water uptake at the end of the experimental period. As a result there was only a slight total withdrawal of water from zone II and even a slight total water uptake by zone IV. The total amount of water uptake of the whole root will have been about zero. The chloride uptake of zone I is not influenced by the treatment but in zone III we see an increased chloride uptake. Repetition of this experiment gave the same results. For the interpretation we may refer to the increased salt uptake following an increase in the suction tension of the outer solution (Brouwer, 1953*b*, fig. 9) and the increase in water conductivity (Chapter III, fig. 3) by the application of a sugar solution in the medium.

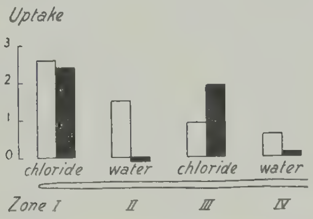

Fig. 7. Comparison of chloride uptake and water uptake of the various root zones at low suction tension in the xylem vessels without an osmotic counter suction in the medium (white blocks) and with such a counter suction (black blocks). The water uptake during application of the sugar solution is strongly reduced whereas the chloride uptake of zone I did not change and that of zone III increased. Values vide fig. 6.

§ 4. Influence of inhibitors on water uptake and anion uptake

These experiments were performed at high suction tension in the xylem vessels. The uptake values from a nutrient solution with and without inhibitors were compared. We have investigated the effect of 2,4-dinitrophenol and oxygen deficiency on the chloride and phosphate uptake. For the chloride uptake a 5 mM calcium chloride solution was used; for the phosphate uptake a Hoagland solution with labelled phosphate.

From all the experiments it appeared that the anion uptake was much more sensitive to a treatment with inhibitors than the water uptake. The results of two experiments with 2,4-dinitrophenol (10^{-5}M) are given in table IX (IX *A* chloride; IX *B* phosphate). In the latter case the inhibitor is only added to the medium of zones II and III.

TABLE IX

Influence of 2.4-dinitrophenol, 10^{-5} M, on the water and anion uptake at high suction tension in the vessels. Water uptake in mm³, chloride uptake in γ Cl per 24 hours per 3 cm root length. Application of inhibitor indicated by italics.

A (chloride)			B (phosphate)		
Zone			Zone		
IV_{water}	730	*746*	$\text{IV}_{\text{phosphate}}$	6	5
$\text{III}_{\text{chloride}}$	40	*18*	$\text{III}_{\text{water}}$	640	*655*
II_{water}	560	*540*	$\text{II}_{\text{phosphate}}$	11	*6*
$\text{I}_{\text{chloride}}$	31	*12*	I_{water}	470	455

TABLE X

The influence of oxygen deficiency on the water and chloride uptake of the different root zones at high suction tension in the vessels. Uptake in mm³ water and γ Cl per 24 hr per 3 cm root length

Zone	Aeration		
	on	off	on
IV_{Cl}	37	23	35
$\text{III}_{\text{water}}$	580	600	570
II_{Cl}	39	18	38
I_{Cl}	32	11	24

In both cases the water uptake was not inhibited, but the anion uptake was strongly inhibited. These data strongly confirm the results of the preceding chapter.

The reversible inhibition caused by stopping the aeration of the zone vessels is shown in table X. It appears that the anion uptake is extremely sensitive to this oxygen deficiency while the water uptake, as expected on the ground of many preceding experiments, does not decrease. The inhibition of the anion uptake by a period with oxygen deficiency is probably not fully reversible for the tip zone.

§ 5. DISCUSSION

The results of the experiments of this chapter confirm the conclusions which were drawn from the experiments of the preceding chapter. The most striking result is that on the one hand there was a distinct correlation between water and anion uptake, on the other hand it was possible to separate both processes. By means of inhibitors it is possible to reduce the anion uptake without influencing the water uptake. Moreover, an osmotic counter suction in the medium diminishes the water uptake without reducing the anion uptake. It seems, therefore, that the total anion uptake, including the increased anion uptake at an enhanced suction tension in the xylem vessels, is a fully active process. These experiments do not indicate that there is any passive transport of ions from the medium to the xylem vessels in the transpiration stream.

It seems appropriate to record briefly the results obtained so far.

a. by increasing the suction tension in the xylem vessels an enhanced water uptake and an enhanced anion uptake take place;
b. the water uptake increases in all zones, most strongly, however, in the basal zones;
c. the anion uptake of the root tip does not increase, that of the other zones does;
d. the increase in anion uptake is proportionally strongest in the basal parts;
e. the anion uptake increases to a lesser degree than the water uptake;
f. the increased anion uptake did not prove to be caused by a passive transport in the transpiration stream.

Each of these facts is important and will be discussed more extensively in the general discussion. In particular, it is interesting to know the nature of the relation between water uptake and anion uptake. To get an answer to this question we have to consider the nature of the salt uptake mechanism. The results of the experiments of Hoagland, Steward, Lundegårdh, Robertson and their co-workers, suggest the desirability of studying respiration in connection with the observed phenomena.

Chapter vi

THE RESPIRATION OF THE DIFFERENT ROOT ZONES

§ 1. Introduction

In the literature several experiments are described concerning the respiration of the different root zones. The methods used differ strongly, viz. from a reduction of methylene blue absorbed (Lund and Kenyon 1927) to a determination of the oxygen consumption in Warburg vessels (Machlis, 1944*b*). The material used also differred. But all authors agree in stating that the oxygen consumption of the tip is much greater than the oxygen consumption of the more basal parts.

Only a few experiments deal with the difference in oxygen consumption in distilled water and in a salt solution. Lundegårdh (1940) has shown that respiration in a salt solution is stronger than in distilled water. Using excised roots he found the strongest respiration occurred in the tip segments (0–3 cm from the tip). The respiration of the following zones, 3–6 and 6–9 cm from the tip, amounted to about one third of this value. With intact plants Gregory and Woodford (1939), using the main root of *Vicia faba*, found a similar gradation from tip to base. The experiments described below show the oxygen absorption of the different root zones from the outer solution, both with high and low water uptake, in distilled water and in a salt solution. It should be considered that only the oxygen uptake from the medium is determined. There might be an oxygen supply from the shoot via the intercellular spaces in the cortex. If so, it is not an important one, for it appeared that stopping the aeration of the outer solution caused a strong reduction of the anion uptake. VLAMIS and DAVIS (1944) showed that the salt uptake of roots which are provided with oxygen from the shoot is independent of aeration of the medium. After cutting off the shoot the salt uptake was dependent on aeration. It is likely, therefore, that in the apparatus used the roots of *Vicia faba* do not obtain oxygen from the shoot.

The method followed has been described in chapter II.

§ 2. RESPIRATION OF THE VARIOUS ROOT ZONES IN DISTILLED WATER AT LOW AND HIGH WATER UPTAKE

In these experiments the respiration of the different zones at low and high values of water uptake are compared under conditions of constant light and temperature. As communicated elsewhere (BROUWER, 1953*a*) no difference in respiration intensity was found. This appears from the results which are plotted in figure 8. This observation agreed with the results of KRAMER and WILSON (1949), LOWENECK (1930) and others. One can conclude from these experiments that there is no correlation between water uptake and oxygen consumption. The oxygen consumption of the tip zone is always stronger than the oxygen consumption of the more basal root parts.

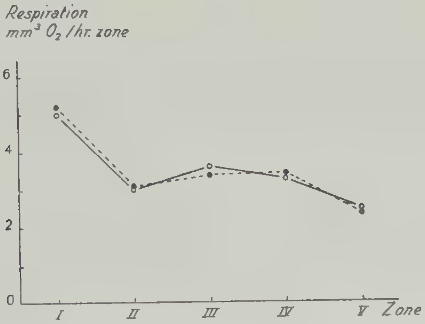

Fig. 8. The oxygen uptake of the different zones from the medium at low transpiration (solid line) and at high transpiration (dotted line).

§ 3. RESPIRATION IN A SALT SOLUTION AS COMPARED WITH RESPIRATION IN DISTILLED WATER

A number of investigators have shown that the presence of ions in the outer solution has an influence on the level of respiration. Working with wheat roots Lundegårdh and Burström (1933) were the first to mention this phenomenon. Thereafter, various investigators (Van Eijk, 1939; Steward and Preston, 1941; Robertson and co-workers, 1941 cf.) observed it with other material. The difference between respiration in a salt solution and respiration in distilled water was called salt respiration or anion respiration.

It appeared that the different zones of the root of *Vicia faba* also showed such a salt respiration. At first the respiration of the different root zones was measured after a long period (16 hours) in distilled water. Immediately after this determination the distilled water was replaced by a 10 mM potassium nitrate solution and the respiration was measured again. In all cases the level of respiration was then higher. An example of such an experiment is plotted in figure 9. Not only is the level of respiration in distilled water highest in the apical region, but also the difference between respiration in a salt solution and respiration in distilled water is highest in this region. The difference between these two levels is plotted in fig. 9 as salt respiration. Expressed as percentages of the respiration in distilled water the salt respiration in *Vicia faba* roots is much smaller than that found by Lundegårdh for wheat roots.

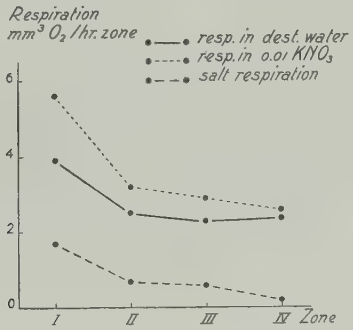

Fig. 9. The oxygen uptake from the outer solution of the different root zones in distilled water and in a 10 mM KCl solution. The difference between these two values is plotted as salt respiration.

§ 4. Salt respiration at low and increased salt uptake

Milthorpe and Robertson (1948) showed that for barley roots a long period elapsed before the salt respiration ceased after transferring the roots from a salt solution to distilled water. Also with my material there was an after effect which lasted for some hours. To compare the salt respiration at a low suction tension in the vessels with that at a high suction tension in the xylem vessels the experiments were performed in the following way.

After a pretreatment in distilled water (16 hours) the salt respiration at low suction tension in the xylem vessels (tap water in the main vessel) was determined as indicated in § 3. Thereafter the salt solution was replaced by distilled water. At the same time the tap-water in the main vessel was replaced by a 2.5 atm Brenner solution and after 6 to 8 hours the respiration in distilled water was again determined. The level of this respiration appeared to be nearly as high as with the former determination in distilled water. During the next period the respiration in a 10 mM potassium nitrate solution was measured. In spite of repeated efforts no difference between the salt respiration at low and at high suction tension in the xylem vessels could be demonstrated. The values for salt respiration of the different zones are plotted in figure 10. The black dots represent the salt respiration at low suction tension, the open dots the salt respiration at high suction tension. We have shown in the preceding chapters that the salt uptake was considerably higher in the latter case. This is a remarkable fact, for we know that it is likely that the anion uptake, even with increased water absorption, is an energy consuming process.

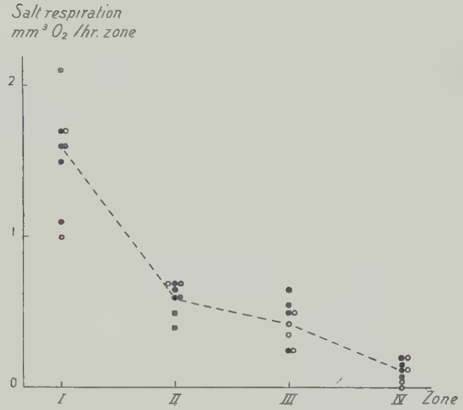

Fig. 10. The salt respiration (difference between respiration in 10 mM KNO_3 and in distilled water) of the various root zones at low and high water uptake. The values found in 4 experiments have been plotted. Black dots represent the salt respiration at low water uptake, open dots the salt respiration at high water uptake.

§ 5. Discussion

From the results described above, it is clear that respiration is highest in the apical region. The values found are expressed per unity of root length, for the roots used have a uniform diameter over the whole length. The fresh weights per centimeter of root length, therefore, differ only slightly.

As compared with other investigations the tip zone in these experiments is very long, viz. 25 to 30 mm, so that it seems likely that it can not be considered uniform tissue with regard to respiration activity. On the contrary, some difference can be expected between the extreme tip and the rest of the tip zone. At distances greater than 30 mm from the tip respiration is more or less uniformly distributed and diminishes only at about 12 to 15 cm from the tip.

The higher respiration of the tip zone must be ascribed to the presence of a differentiating tissue and, therefore, consists partly of growth respiration.

Further, it is possible to show a salt respiration with *Vicia faba*. It is not surprising that the salt respiration found here is relatively smaller than that of wheat roots, because the former are much thicker and show a smaller salt uptake than grasses.

As stated in the introduction it has not been proved that salt respiration provides the energy for salt uptake. According to the strong correlation between salt respiration and salt uptake LUNDEGÅRDH assumes that this correlation is causal. Such an interpretation is accepted by most investigators.

If we follow this view, it can be concluded from the results given, that the ratio between the number of ions taken up and the oxygen consumed is not a constant one. The amounts of ions taken up at low suction tension and at high suction tension differ distinctly, the oxygen consumption being the same in both cases. In table XI a comparison of the ratios between anion uptake and anion respiration at low and at high suction tension is given. The values recorded in this table are average figures for 8 experiments, both for salt respiration and salt uptake. Salt respiration and salt uptake were determined with different plants. The salt solution used was in both cases a 10 mM potassium nitrate solution. Given the uniformity of the material used we can expect a reasonable picture from these comparative experiments.

TABLE XI

Comparison of the ratio $\frac{\text{milliaeq. NO}_3\text{ absorbed}}{\text{milliaeq. O}_2\text{ respired}}$ at low and high water uptake for the different root zones

Zone	low water uptake			high water uptake		
	m. aeq. O_2	m.aeq. NO_3	Ratio	m. aeq. O_2	m.aeq. NO_3	Ratio
IV . . .	0.32	0.23	0.7	0.31	0.74	2.4
III . . .	0.36	0.33	0.9	0.36	0.90	2.5
II . . .	0.45	0.37	0.8	0.46	1.05	2.3
I . . .	0.80	0.56	0.7	0.78	0.62	0.8

In the first place it is striking that the efficiency of the salt respiration of the different zones, which does not essentially differ at low suction (column 3), does differ at high suction tension. In the latter case (column 6) the efficiency of the apical zone has not changed, while the efficiency of the basal zones is strongly increased. Thus, an increased suction tension in the vessels renders the anion uptake easier which means that with the same expenditure of energy more ions are taken up.

Though not comparable with this phenomenon ROBERTSON found that the efficiency of salt respiration increased with rising concentration of the nutrient solution untill a maximum was reached. This followed from the fact that the relation between salt uptake and salt concentration differed from the relation between salt respiration and salt concentration.

The above reasoning stands or falls on the question of whether or not the salt respiration causes the salt uptake. The experiments of investigators who are engaged in this problem, moreover, have a bearing on the accumulation in the tissue itself, rather than on the total uptake, that is the sum of the amount withheld in the root tissue and the amount transferred to the shoot. It is of great importance, therefore, to trace to what extent these two processes occur.

Chapter VII

UPTAKE AND BINDING IN THE TISSUE

§ 1. Introduction

Little difference is made in the literature between accumulation and total absorption, though we may assume that there is a great difference between these two processes, especially with regard to the roots. Conclusions as to the absorption capacity are often based on experiments on the accumulation in the tissue. I am not aware of any attempt to compare the relation between these two processes, accumulation and uptake, with regard to the distribution along the root axis.

To make such a comparison it would be advantageous to use ions, which are not present in the root at the start of the experiments. Most investigators have used ions like bromide or radio-active isotopes.

Since it was necessary to determine an amount of plant material as small as 1 cm of a single root, labelled phosphate (P*) was chosen for the experiments described below. It can be estimated in small amounts. A drawback is that in addition to an active binding a more or less passive exchange against phosphate already present in the tissue, may probably occur. In the experiments described below the total amount of P* present in the tissue was determined and no correction for such an exchange was made. Hence the actual amounts, actively taken up by the root tissue may be somewhat lower than the amounts found in the experiments.

§ 2. Method

The plants were mounted on the apparatus in the usual way. The uptake periods lasted 24 hours. During these periods a full strength Hoagland solution with P* served as a nutrient solution in the zone vessels. The total uptake by the different zones was determined as described above. After the uptake period the root zones were rinsed with distilled water to remove loosely adsorbed phosphate. Thereafter, the zone vessels were removed and the root was cut into 1 cm segments. These segments were placed on small planchets and allowed to dry. After drying the activity of each was determined and compared with the activity of a small drop of the nutrient solution of known weight. The latter was also placed on the planchet and evaporated to dryness. The experiments were performed partly at low and partly at high suction tension in the xylem vessels.

Further, a number of experiments were performed in which labelled phosphate solution was applied to a single zone and a Hoagland solution without P^{32} to the other zones. The activity of all segments was determined in order to get an impression of the transport in the root tissue itself.

§ 3. THE RATIO BETWEEN TOTAL UPTAKE AND THE AMOUNT WITHHELD IN THE TISSUE

From the experiments it appeared that there was a great difference between the growing tip and the rest of the root. The occurrence of growth seemed to be particularly important. For this reason the growth was measured in each experiment in addition to the uptake and the binding in the root tissue. This can be easily performed with the apparatus used. An example of the uptake and the binding by the root is given in table XII.

TABLE XII

The ratio between phosphate uptake and phosphate binding of the different zones of the root at low water uptake. During the experiment the length of the tip zone increased from 25 to 40 mm

Zone	Uptake	Binding	% transfer
III	6.1 γ P	0.89 γ P	86
II	8.2 ,,	0.99 ,,	88
I	12.3 ,,	5.9 ,,	52

At the start of the experiment the length of the tip zone amounted to 25 mm, at the end, i.e. after 24 hours, it amounted to 40 mm. The mean length during the uptake period was $(25 + 40)/(2) = 33$ mm. The total uptake amounted to 12.3 γ P, or calculated per 3 cm root length, 11.2 γ P per 24 hours. The activity determinations showed that in the tip itself 5.9 γ P was present (per 3 cm root length 4.4 γ P). Thus, of the total uptake 48 % $((5.9 \times 100)/(12.3))$ was bound in the root tissue and 52 % was transferred to the shoot or the other parts of the root system. For zone II we found a binding percentage of 12 %, whereas 88 % was transferred. Zone III showed 14 % bound and 86 % transferred.

This experiment was performed at low suction tension in the xylem vessels. The ratio between binding and transfer as found here for the tip was about the average value for the experiments performed. In a single case we found that the amount withheld by the tissue of the root tip was about 83 % of the total uptake. On the other hand a non-growing tip showed about the same ratio as the rest of the root. It can be concluded that the ratio between binding and uptake of the apical zone is strongly dependent on its original length and growth.

At a higher water uptake the ratios between uptake and binding

TABLE XIII

The ratio between phosphate uptake of the different root zones and phosphate binding in the tissue at high water uptake. Tip zone showed no growth

Zone	Uptake	Binding	% transfer
IV	10.4 γ P	0.6 γ P	94
III	water uptake determ.	0.01 ,,	—
II	8.8 ,,	1.1 ,,	88
I	7.0 ,,	1.05 ,,	85

are somewhat different depending on the amounts of ions taken up. An experiment in which the uptake was rather low is given in table XIII. The root tip showed no growth in this case. The phosphate uptake of zones I, II and IV was determined from a Hoagland solution with P*. Zone III (5 cm long) was used for determining the water uptake from a Hoagland solution without P*. We see that the phosphate uptake of zone IV is highest, the binding in the root tissue being lowest at the same time. The binding in zone II does not differ essentially from that of zone II of table XII. It is a striking fact of all these experiments that the amount of ions withheld in the tissue is rather independent of the rate of water uptake. At high suction tension a higher percentage of the total uptake is involved in transfer to the shoot.

Moreover, there appears to be only a small amount of P* withheld in zone III, which was not in direct contact with the labelled phosphate solution, though about 13 γ (difference between uptake and binding of zones I and II) must have passed through the vessels of this zone. This phenomenon will be further analysed in the next section.

§ 4. Longitudinal transport in the root

In the preceding section the binding by the tissue of each zone was given. Seeing that the length of the tip zone varied from 25 to 40 mm the distribution must be further analysed. By making the partition as small as possible a difference between the various parts of the apical zone can be observed. In figure 11 *A* the distribution of the amount of phosphate withheld per cm root length is given. The series of numbers right and left show the results of one experiment each. The apical 10 cm (shaded) of these roots were able to absorb phosphate from a Hoagland solution with P* during 24 hours. The more basal parts, 11 to 16 cm inclusive and 11 to 13 cm inclusive from the apex, were in the main vessel in tap water. The numbers beside the root in fig. 11 *A* refer to the counting-activity (1000 countings corresponds to 1.07 γ P). It appears from these data that most phosphate is withheld by the apical 1 cm, and from there to the base a gradually decreasing amount of phosphate is withheld. Those parts of the root which were not in contact with the P^+ solution held only very small fractions of these amounts, though the whole amount that is taken up by the lower parts and that is not withheld there, must have passed through the vessels of these parts of the roots. So it seems that there is only a small exchange between phosphate present in the vessels and that in the tissues.

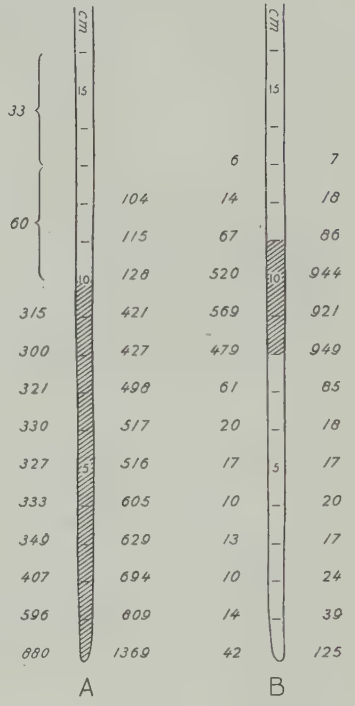

Fig. 11*A*. The distribution of the activity (counts per minute) of dried, 1 cm long segments, along the root axis after an uptake period of 24 hours. During this period a Hoagland solution with labelled phosphate served as nutrient solution for the apical 10 cm of the root (shaded). The more basal parts were in tap water. The countings left and right represent the results of different roots. (1000 counts = 1.07 γP)

Fig. 11*B*. The distribution of the activity (counts per minute) of dried, 1 cm long root segments along the root axis, after an uptake period of 24 hours during which zone IV (shaded) was able to absorb phosphate from a Hoagland solution with labelled phosphate. The other root parts were in a Hoagland solution without P^{32}. The countings left and right represent the results of different roots. (1000 counts = 0.6 γ P)

Figure 11*B* shows the distribution of phosphorus (two experiments also) absorbed by zone IV (9–11 cm inclusive from the apex) which was the only zone that was allowed to absorb the labelled phosphate. Zones I, II, III and V were placed in a Hoagland solution without P*. At the end of the experiment the whole root was divided into 1 cm segments and, after drying, the activity was determined. It appears from the data that the treated zone shows the normal picture, or a piling-up of about 0.7 to 1.5 γ P (1000 counts/min = 0.6 γ P). The first cm bordering on this zone towards the root apex as well as towards the base, shows an activity of about $^1/_{12}$ of the treated zone, whereas the zones further removed have an activity of about $^1/_{50}$ of this zone. The utmost tip makes an exception, showing a distinctly greater activity than the rest of the root not in contact with the P^+ solution.

How can these results be explained?

It seems to be most obvious to assume that the high activity of the root parts bordering the treated zones are due to a diffusion in the cortex. The large influx of phosphate ions, however, will enter the central cylinder where an upward transport in the xylem vessels and a downward transport to the tip will take place. Nothwithstanding the fact that the apical zone is able to absorb phosphate from the medium, it shows a distinct piling-up of phosphate absorbed in the more basal region.

In connection with the anatomical proportions of the root, impermeability of the endodermis etc., it is noteworthy that the ions taken up by a distinct zone penetrate the endodermis. LUNDEGÅRDH and also KRAMER assumed that a longitudinal transport through the cortex in the direction of the tip takes place after which a transfer to the stele occurs in the apical region. This concept is not supported by these experiments. Given a somewhat important transport through the cortex in a longitudinal direction we should expect that the activity of the root segments would gradually decrease from the place of application of the active nutrient solution along the root in both directions.

The data of figure 11*B* are obtained by cutting the root in 1 cm sections. If we take shorter segments it appears that the radiation is limited to the first 3 mm on both sides of the treated zone. This renders a longitudinal transport in the cortex unlikely.

§ 5. RELEASE AND EXCHANGE OF PHOSPHATE

If we determine in an experiment as described above the activity of the outer solution of the zones which have not been in direct contact with the P* solution, it appears that no P* is released by those parts of the root. The ions present in the stele show no exchange with the phosphate present in the medium. The phosphate transported to the apex of the root, to the base of the root or to the shoot is protected against leakage and exchange.

§ 6. DISCUSSION

The experiments described in this chapter are interesting in various respects. Firstly, it is clear that the ions absorbed are transported for the greater part to the shoot. On the other hand it appears that the distribution of the binding in the tissue can not be used for conclusions on the absorption capacity. Uptake by and consequent binding in the tissue are two different processes.

Further, it seems to be obvious that the ions absorbed by a distinct root zone are mainly transported to the stele in radial direction. KRAMER and WIEBE, who investigated the accumulation of P in barley roots, found that this accumulation was highest in the root hair zone and that it decreased in apical as well as in basal directions. As in the experiments described here, they showed that with an uptake limited to the growing tip, no transport to more basal parts occurred. The total uptake was withheld in the tissue of the growing region. An uptake by the root hair zone showed a transport both in the direction of the root tip and in the direction of the base. No exact data are given, however, as to this subject.

From the fact that in our experiments such a rapid fall of the P* content of the root tissue occurs upwards and downwards from the zone of application, it can be assumed that longitudinal transport in the cortex meets with considerable resistance. It is likely that this holds good both for salt transport and for water transport. For that reason an upward current of ions in a longitudinal direction through the cortex, as suggested by HYLMÖ, seems to be unlikely.

Further attention must be drawn to the relation of the distribution of binding in the tissue and salt respiration along the root axis. Thus, it appears that salt respiration is running parallel to both uptake at low suction tension and binding in the tissue. We have to be careful, therefore, with our conclusions on the connection between salt uptake and salt respiration. It is not inconceivable that salt respiration is only responsible for the piling-up in the tissue, as occurs in storage tissues, and that on the other hand the transport of ions from medium to vessels is based on another mechanism.

The results of these experiments on phosphate binding in the tissue agree fully with the bromide accumulation described by PREVOT and STEWARD for the same material. It seems justified, therefore, to attach some general meaning to these phosphate experiments.

CHAPTER VIII

THE ANATOMY OF THE ROOTS

§ 1. INTRODUCTION

A survey of the anatomy of roots in connection with the problem of uptake from the surroundings can be found in a paper of WIERSUM (1947). On the basis of the previous literature he divides the root from tip to base, in three zones, viz. the undifferentiated tip zone, the more or less differentiated zone with root hairs and with an endodermis in its primary or secondary stage, and the basal root zone which is covered by an isolating suberized layer.

It is reasonable to suggest that there is no uptake of water or salts in the latter zone because of the presence of this tightly fitting layer of suberized cells. As soon as lenticells are present, it is possible for water and solutes to enter the root. ADDOMS (1946) and also KRAMER (1946) showed that this really happens. For the normal water and salt uptake this zone will be of little importance.

If we consider the undifferentiated tip zone, no anatomical features are apparent which can be considered to prevent water and ions from entering this part of the root. The growing cells present here can easily provide themselves with salts and water. The uptake will be limited, however, by the direct requirements of the cells.

Between the two zones mentioned lies the zone with fully differentiated tissues and without a suberized surface layer. This is the zone where root hairs normally occur. An uptake of water and salts from the medium is possible. The possibility of a transport to the stele is dependent on the developmental stage of the endodermis. A radial transport through the walls is unlikely because of the presence of

Casparian strips in the radial walls of the endodermal cells. Transport through the protoplasm is the only possible alternative.

Finally, the endodermis in the secondary stage is fully suberized, with the exception of the passage cells which remain in the primary stage.

Of course, the zones mentioned above are not clearly separated. There exist conversions of which, in particular, that between the undifferentiated tip and the differentiated parts is of special interest. This region constitutes that part of the root in which a transport to the xylem vessels begins.

§ 2. Anatomy of the roots used for the experiments

The anatomy was investigated on long, unbranched, secondary roots which were used for the experiments. Transverse, tangential and longitudinal free-hand sections were used, representing segments at different distances from the root tip. The roots investigated were grown in tap water. The root tip is protected by a root cap. The tip itself, 1 to 3 mm, contains small isodiametrical, parenchymatous cells and no tissue differentiation can be observed.

The greater the distance from the apex, the greater the diameter of the cortex cells, whereas the cells of the central cylinder show only a small enlargement in diameter but an important increase in length. All these features refer to the first few mm of the root apex. At a distance of about 4–5 mm from the tip the first xylem elements develop and a distinct difference between cortex and stele becomes visible. The extension of the cells and a further differentiation takes place between 4 and 7 mm from the tip. The transition to a more or less complete differentiation occurs in the region between 7 and 10 to 15 mm from the tip. A distinct wood reaction can be demonstrated there.

At a distance of 10 to 15 mm from the tip the endodermis can be noticed, but a suberisation of the radial walls does not yet exist. Casparian strips can not be found. In the cortex the first intercellular spaces originate. The epidermis is colourless.

At a distance of about 20 mm from the tip the tissues are fully differentiated. The largest cells of the cortex are situated in the outer layers, more inwardly the cells become smaller. The endodermis can clearly be identified by the suberisation of the radial walls. In the central cylinder the vascular bundles are fully developed. The rest of the tissue of the stele consists of long stretched cells with a small diameter.

This state exists over the whole root length up to the insertion on the main root. The epidermis becomes yellowish. The endodermis remains in its primary stage. At greater distancess from the apex, in the longitudinal walls of the endodermis cells thickenings arise which are somewhat suberized, but a complete suberisation of these walls does not occur.

Root hairs exist only at those parts of the root which arise above the liquid.

§ 3. Discussion

The anatomical data described above are in full agreement with the results of physiological investigations. Because of the absence of vascular bundles in the apical 1 cm of the root the uptake of water there is virtually impossible. Salts can be absorbed but only as far as they can be used for growth.

The adjacent differentiating zone, 1 to 2 cm from the apex, will also consume an important part of the ions taken up for its own growth. The experiments described in chapter VII can be easily explained when these physiological factors are considered.

That the endodermis over the whole root length is in its primary stage was not to be expected. This fact, however, makes it clear that a transport in radial direction can take place over the whole root. On the other hand, on account of the anatomical features, we should expect that the uptake of water and salts would occur equally easily over the whole length. The reverse is true, however. Therefore, the existing differences must be ascribed to physiological differences, possibly connected with differences in age.

Chapter IX

GENERAL DISCUSSION

In order to obtain a survey of the experiments and theoretical aspects of this study reference will also be made to the preceding papers of the author. It is a well known fact that the water uptake is determined by the suction tension in the xylem vessels and the water conductivity of the root tissue between the medium and these vessels, as is briefly indicated by the formula of Brieger: Uptake = conductivity × suction tension. If a suction tension exists in the medium, the effective water moving power amounts to: $S_{xylem} - S_{medium}$.

On enhancing the transpiration the suction tension in the xylem vessels increases. If no changes in water conductivity occurred, the uptake should increase in proportion to the increase in suction tension, resulting either from the enhanced transpiration or from a hampered water absorption.

In fact the water uptake increased to a higher degree than the suction tension did. This must have been due to an increase in water conductivity. Brewig showed for the root of *Vicia faba* that the increase in water conductivity was dependent on the region of the root involved. He found that the changes in conductivity occurred especially in the mature root parts and he assumed they were caused by the enhanced suction tension. This is made more probable by the results obtained from the experiments of this investigation (chapter III). Moreover, not only did an enhanced suction tension in the xylem vessels appear to increase the water conductivity but an enhanced osmotic suction tension in the medium had the same effect. This is in accordance with similar findings of Rosene.

From the fact that both an increase in suction tension in the xylem vessels and an identical increase in osmotic suction tension in the medium cause the same changes in water conductivity of the tissue it can be concluded that the point of attack does not matter. Both suction tensions will cause an increase in suction tension of the cells of the root tissue, these cells being in equilibrium with the surroundings. An increased suction tension in the cells means a decreased turgor pressure. It is supposed that this decrease in turgescence is the direct cause of an increased water conductivity. The latter is not only effective for a water current from medium to xylem vessels but also for a current from the xylem vessels to the medium, if the suction tension of the medium exceeds the suction tension in the xylem vessels.

The water transport itself is a fully passive process. This appears, among other things, from the fact that it is independent of the oxygen supply. Further, inhibitors in concentrations that inhibit the respiration, have no influence on the water uptake. The fact that potassium cyanide in higher concentrations (5.10^{-3} and 10^{-3}) decreases the water uptake (at the concentration 5.10^{-3} directly and completely) does not prove that an active portion of the water uptake process is eliminated. We have to assume that as a result of these high concentrations the conductivity of the tissue between medium and xylem vessels decreases and after some time becomes infinitely small, so that no water uptake at all can take place. Presumably this will be due to changes in the structure of the protoplasm.

With chloroform we get the reverse of this process, viz. a temporary increase in conductivity. (BROUWER, 1953).

An excess of carbon dioxide in the medium causes an initial decrease followed by an increase in conductivity.

These facts, supported by many investigations of other physiologists (vide KRAMER, 1932 cf.), argue in favour of a complete regulation of the conductivity by the protoplasm.

It must be left an open question whether this regulation of the conductivity takes place in the endodermis or over the whole pathway between medium and xylem vessels.

From the above it is clear that the changes in water conductivity must take place in the protoplasm. From the correlation between the turgescence of the tissue and water conductivity it may tentatively be assumed that the pressure upon the protoplasm between vacuole and cell wall is an important factor. A decrease of this pressure makes possible a certain expansion of the protoplasm that is perhaps coupled with a change in hydration, thus causing an increased water conductivity.

Another problem closely related to this question is that of different reactions to an increased suction tension in the various root zones. We have seen above that the water conductivity of the apical zone does not increase in this case whereas that of the more basal zones does. It appears that the changes in water conductivity are inversely proportional to the turgor stretching of the zones. This means that the tip which does not show any changes in water conductivity possesses the greatest turgor stretching. On the contrary, the basal parts have only a little elastic cell wall and in these very zones the changes in water conductivity are greatest. In these older zones a water withdrawal from the vacuole will not be followed by a decrase in volume of the cell to such an extent as will be the case in the apical region. The consequence of this fact is that a reduction of the pressure on the protoplasm occurs primarily in the mature root parts.

Much attention has been called to the changes in water conductivity, and not without good reason, for the effect of these changes for both water and salts is very similar.

The experiments on salt uptake were started with the intention of gaining an insight into the distribution of the uptake along the root axis. In this respect most of the experiments described in the literature give only an impression of the accumulation in the root tissue. Though many conclusions as to the uptake by the root are based on this accumulation they do not seem to be justified (Arisz, 1945). It appears that the anion uptake at low water uptake is greatest in the apical region, and rather small in the basal zones. The binding in the tissue, though on a much lower level, shows the same gradient from tip to base. At high water uptake, however, we get the same binding gradient but the total uptake has another distribution along the root axis. The amount of anions taken up by the tip does not change but that in the more basal zones increases in an inverse proportion to their distance from the tip. The maximum anion uptake at high water uptake lies in zone II or III. More basal zones show the relatively strongest increase but the total level does not reach that of zone II or III, because of the low ion uptake at low water uptake. Thus, the distribution of the anion uptake at high water uptake deviates from the distribution of the binding capacity.

With phosphate we have examined what portion of the total uptake was withheld by the root tissue and what part was transferred to the shoot. It appeared that the growing root tip fixed a rather great part of the total phosphate absorption varying from 30 to 80 %, whereas this percentage was much lower in the other zones. As to the distribution along the root axis the binding of phosphate as determined in these experiments, and which is assumed to take place mainly in the protoplasm, strongly resembles the bromide accumulation found by Prevot and Steward with the same material.

If we first consider the anion uptake at low water uptake the question can be asked why the uptake of the apical zones is so much greater than the uptake of the basal zones. Anatomically only the uttermost root tip is different from the rest of the root. It seems likely that the differences between the zones are physiological in nature. If we consider the respiration of the different zones it appears that the same gradient is found. The same holds good for salt respiration. A disparity of age may be responsible for the differences.

After increasing the water uptake (the anion uptake also increases) the distribution of the anion uptake more or less resembles that expected on the basis of the anatomical structure. The differences between the various zones almost disappear. This is in contrast with what is found with the water uptake where at high suction tension a steep gradient of the water absorption value is found from base to tip.

The influence of the suction tension on the water uptake by the different zones on the one hand and on the nitrate, chloride and phosphate uptake on the other hand can be compared by means of figure 12. It appears that the water uptake increases more than the anion uptake. This is due to an increase in the water uptake owing to an increased suction tension and an enhanced conductivity. If, however, a correction is made for the difference in the driving force, i.e. if the water conductivity values are compared instead of the water absorption values, a totally different picture is obtained. In figure 12 that part of the increased water uptake that is due to increased suction tension in the xylem vessels is recorded above the dotted line.

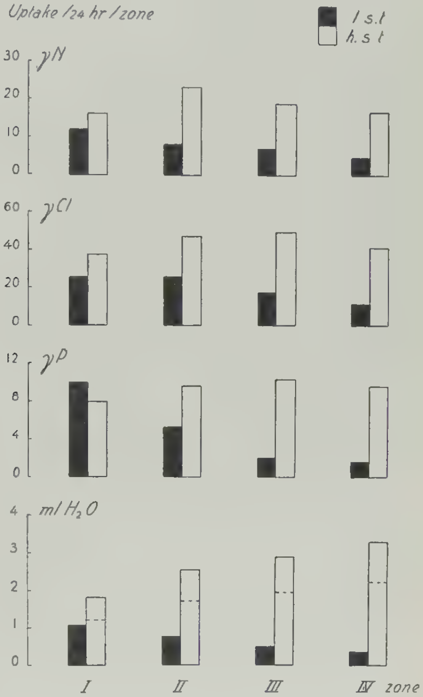

Fig. 12. The nitrate, chloride, phosphate, and water uptakes of the different root zones at low (l. s. t.) and high suction tension (h. s. t.) in the vessels. In the columns representing water uptake at high suction tension (white blocks) the amount above the dotted line is due to the increase in suction tension, the difference between black and white blocks up to the dotted line being due to an increase in conductivity.

It appears that the ratio $\frac{\text{white}}{\text{black}}$ for anions and the ratio

$$\frac{\text{white up to the dotted line}}{\text{black}}$$

for water for the different zones is about the same. This means that on increasing the suction tension in the xylem vessels the anion uptake increases to the same extent as the water conductivity.

In spite of extensive investigations on the mechanism of the salt uptake, for which we may refer to the recent reviews of ARISZ (1945), HOAGLAND (1948), BROYER (1951), ROBERTSON (1951), BURSTRÖM (1951), OVERSTREET and JACOBSON (1952) and SUTCLIFF (1953) it is not yet sure how the energy transfer from respiration to ion uptake is brought about. It is certain, however, that this energy is supplied by respiration, possibly only by the salt respiration. Both total respiration and salt respiration proved to be equal at low and high suction tensions in the xylem vessels, and there seems to be no reason, therefore, to assume that the energy available for salt uptake is greater at high suction tension than at low suction tension. If this is true, this process of uptake is more effective at high suction tension than at low suction tension, or in other words the resistance against salt transport is lower. It seems, therefore, that enhancing the suction tension in the xylem vessels causes a similar increase in conductivity both for water and anions, as a result of a decreased turgescence of the tissue. It is not surprising, therefore, that increasing the suction tension in the medium at constant low suction tension in the xylem vessels, does increase the anion uptake (figure 7).

One assumption for this view is that the salt uptake at high as well as at low suction tension is a fully active process. This seems probable from the experiments on the influence of inhibitors and countersuction. Both methods have also been applied by other investigators. In particular inhibitors are often applied to get a better view of the mechanism of uptake processes (ARISZ, 1953; LUNDEGARDH; ROBERTSON). High concentrations, however, are always injurious, but even the very low concentrations used here, caused an inhibition that was not completely reversible, probably because of the long periods of application. Withholding oxygen from the medium showed the same inhibition, however, and appeared to be reversible.

Any after-effect of the application of the sucrose solution which was used for counter suction was, in contrast to a mannitol solution, not noticeable.

These experiments present a new point of view on the influence of transpiration on the salt uptake. To check the general validity of this view, the experiments were repeated with a number of different plants. It appeared that with increased transpiration, in general, the water uptake increased more than the anion uptake. This phenomenon was also found by PETRITSCHEK (1953) with *Clematis*. Using inhibitors and counter suction it was possible to separate water uptake and salt uptake from one another. It appeared that there was no reason to suppose a passive transport of ions in the transpiration stream for the materials used. That is why the interpretation given above is more satisfying. It remains possible, however, that there is a passive transport as HYLMÖ suggested. Unpublished experiments of the author with HYLMÖ's material, *Pisum sativum*, showed a rectilinear connection between water uptake and anion uptake. This confirmed the results found by HYLMÖ. Using 2,4-dinitrophenol, however, a definite inhibition of chloride uptake could be found without any reduction in water uptake. A counter suction with sucrose did not give the results expected. In one experiment the water uptake was not influenced at all, while in another the water uptake was irreversibly reduced, accompanied by a reduction of the chloride uptake.

Investigating the influence of water uptake on anion uptake with rye plants, which were pretreated in a different way (Brouwer, 1953*b*), it appeared that the salt content, and in particular, the content of the ion investigated, determines the uptake of this ion to some degree. Helder (1952) showed that in some cases utilisation assigns a limit to the uptake. If this limit is already reached at low transpiration we may expect that an increase in transpiration does not further promote the uptake. It is likely that this is the case if the ion content of the plant is high and the utilisation is limited. Hence a greater uptake by enhancing the transpiration can not be realised.

The resultant uptake from the medium is the difference between the entrance of ions into the root and leakage into the medium. Now the question arises whether the unchanged uptake is the result of a greater entrance into the root, an equally greater efflux from the shoot and subsequent leakage into the medium, or of an unchanged entrance of ions. The efflux from the shoot is discussed by Alberda (1948) and others and was demonstrated by Phillis and Mason and by Moore (1945).

As indicated by the experiments of Hoagland and Broyer the relative importance of the accumulation in the root system is dependent on the salt status. The latter will also influence the reaction of the salt uptake upon an increased water uptake.

Finally it may be stated, that the phenomenon described in this paper can only be expected if the transport of ions in the root tissue is the limiting factor in the uptake process.

A well-rounded picture of all these processes will only be possible if we are able to determine uptake, accumulation, transfer to the shoot and eventually efflux from the shoot at the same time.

SUMMARY

In the above a survey is given of the experiments on the water uptake described in preceding papers. It appeared that the water uptake is dependent on the suction tension in the xylem vessels and the water conductivity of the root tissue between medium and vessels.

Important stress is laid upon the water conductivity of the tissue, this conductivity being not the same under various circumstances. It is shown that the water conductivity increases on enhancing the suction tension in the xylem vessels. As to the different root zones it appeared that in this case the conductivity of the tip zone did not alter, whereas the conductivity of the basal zones strongly increased.

Moreover, such an increase in conductivity could be caused by enhancing the osmotic suction of the outer solution.

Both the increased suction tension in the xylem vessels and the

osmotic suction in the outer solution, cause a decreased turgescence of the root tissue. It is assumed that this decrease in turgescence is the direct cause of the enhanced water conductivity.

Applying various inhibitors in different concentrations it could be stated that water uptake and water transport are completely passive processes. In spite of this the water transport is controlled by the living protoplasm, because of the fact that inhibitors in high concentrations reduced the water uptake. It is assumed that this is caused by changes in the structure of the protoplasm.

Besides, the anion uptake of the various root zones is investigated. An apparatus to make possible these measurements is described.

At low water uptake the maximum anion uptake is found in the apical root zones, the basal zones showing a rather small anion uptake in this case. Increasing the water uptake the anion uptake increases, this increase being most important in the basal zones.

Moreover, on using respiration inhibitors in various concentrations it appeared to be possible to inhibit the anion uptake without reducing the water uptake. On the other hand an osmotic counter suction in the outer solution reduces water uptake without diminishing the anion uptake. Both processes water uptake and anion uptake, therefore, are related to one another, but this is not a direct relation. Comparing the water uptake and the anion uptake of the different root zones at low and high suction tension in the xylem vessels, it appears that the water uptake increases more than the anion uptake. The water conductivity of the different zones, however, increases to the same ratio as the anion uptake.

An explanation of this phenomenon can be obtained via the respiration. The respiration of the different root zones and also the salt respiration is the same at low and high water uptake. Accepting the hypothesis that the respiration delivers the energy for the anion uptake there is reason to assume that the driving force of the salt uptake is the same at low and high water uptake values, therefore, also at low and high anion uptake values. As a consequence of this fact the anion uptake at high suction tension in the xylem vessels is as much facilitated as the water uptake, or in other words the conductivity for water and anions is increased to the same degree. It is assumed that this is a reasonable explanation as to the influence of the water uptake on the salt uptake. Moreover, such an influence can only be expected if the resistance to the salt transport is the limiting factor in the salt uptake process.

A number of experiments are described dealing with the ratio between the total uptake and the amounts bound in the root tissue. The latter is more or less independent of the water uptake.

Anatomical studies show that the whole root up to at least 20 cm from the root tip, has a non-suberized epidermis and an endodermis in its primary stage. The difference between the various root zones, therefore, must be ascribed to physiological features such as a difference in age.

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